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FIGURES IN THE TEXT

57



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A CORRELATION BETWEEN THE HISTOLOGICAL CHANGES AND THE FATE OF LIVING TUBERCLE BACILLI IN THE ORGANS OF REINFECTED RABBITS

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PLATES 10 TO 13

(Received for publication, August 27, 1932)

Since Robert Koch (1) first observed and described the phenomenon that bears his name there have been repeated attempts to elucidate the differences in the reaction to tubercle bacilli of normal and tuberculous animals, with all the implied problems of natural resistance and acquired immunity. Amongst these may be mentioned the work of Lewandowsky, Krause and Peters, Baldwin and Gardner, Jaffé and Löwenstein, Long, and Gardner (2). They conclude that the lesion of reinfection is differentiated from that of the primary infection by the acceleration and intensification of the immediate inflammatory reaction and by the accelerated formation of nodule and tubercle and the abortive nature of these lesions. Kalbfleisch (3) on the other hand maintains that in rabbits only slightly affected with tuberculosis the course of a second infection differs in no way from the course of a primary infection in the conjunctiva, mesentery and skin, and that in extensively diseased rabbits there is only a quantitative difference, observed chiefly in the skin as a more intensive or, less often, as a less intensive reaction in the tuberculous as compared with the normal animal.

The fate of the bacilli of reinfection has also been studied by numerous investigators (4). However no experiments have thus far been reported in which the fate of the living bacilli and the associated histological changes have been studied together in the same reinfected animal. The rôle of inflammation in resistance to reinfection is far from settled.

In recent experiments (5) on the response of the rabbit to a primary infection the fate of living tubercle bacilli cultured from a given tissue was studied in relation to the associated histological changes. It was found that the mononuclear phagocytes in various organs possess an inherently different capacity to destroy tubercle bacilli. If the bacilli fail to be destroyed by these cells they accumulate in the cytoplasm and the greater the number of the microorganisms the more extensive the multiplication of new mononuclear cells by mitosis. When the mononuclears are transformed into mature epithelioid cells and the tubercles have reached their maximum development the bacilli have already undergone extensive destruction and are disappearing. On the basis of these observations the epithelioid cell of tuberculosis may be described as a mononuclear phagocyte that has killed and incompletely digested tubercle bacilli or their products. After multiplication of the tubercle bacilli has ceased regeneration of mononuclears by mitosis becomes less active and Langhans giant cells may be formed from preexisting epithelioid cells. Lymphocytes and granulation tissue surround and permeate the tubercle after the bacilli have been destroyed, and the tubercle retrogresses.

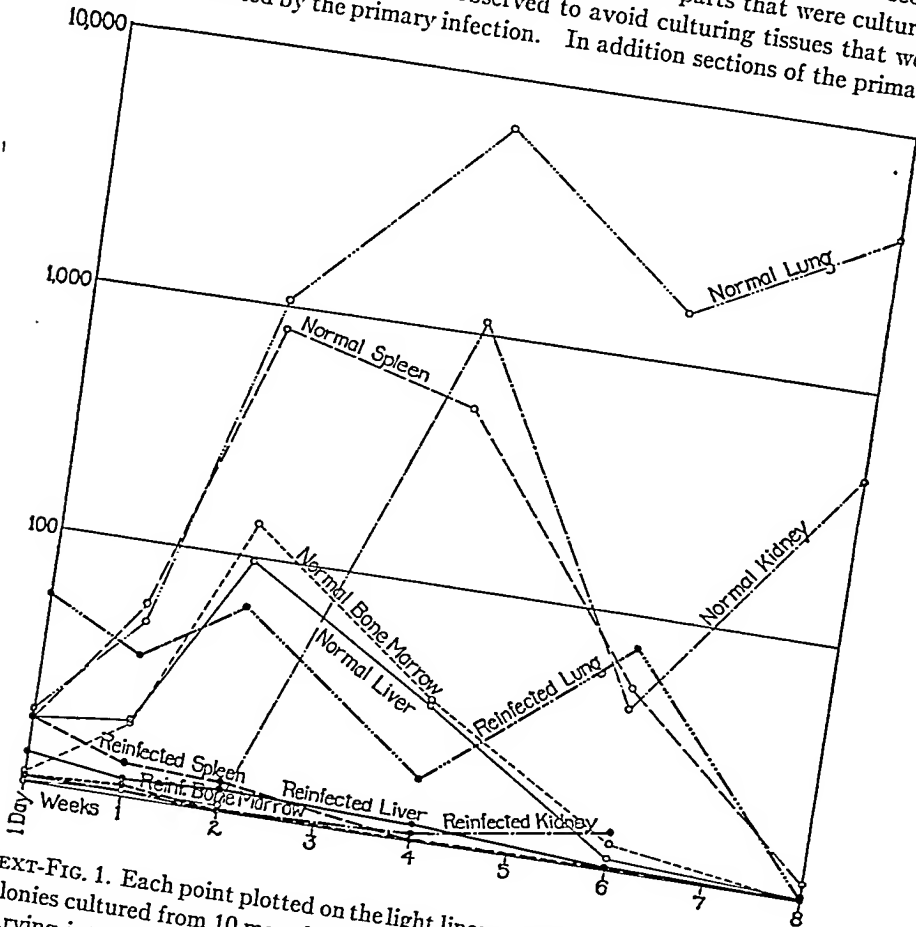
A similar study of the reinfected animal was undertaken to throw further light upon the relation of host and parasite.

Method

A series of rabbits was inoculated intravenously with 0.001 mg. of tubercle bacilli of a human strain (P 48A) per kilo of body weight. It has been shown (6, 1928) that 6 months after such an inoculation the tubercle bacilli have almost completely disappeared from the liver, spleen and bone marrow and have as a rule been greatly reduced in numbers in the lung and kidney. At about this time, therefore, 15 of these rabbits were given intravenously a reinfesting dose of 0.01 mg. of the same strain of tubercle bacilli. Another series of 15 rabbits was infected with the human strain and reinfected with a bovine strain, Bovine C, in the same quantities and by the same route. At the same time two series of normal rabbits, 13 in each, were injected intravenously with 0.01 mg. of these strains, one with the human, and one with the bovine type bacilli.

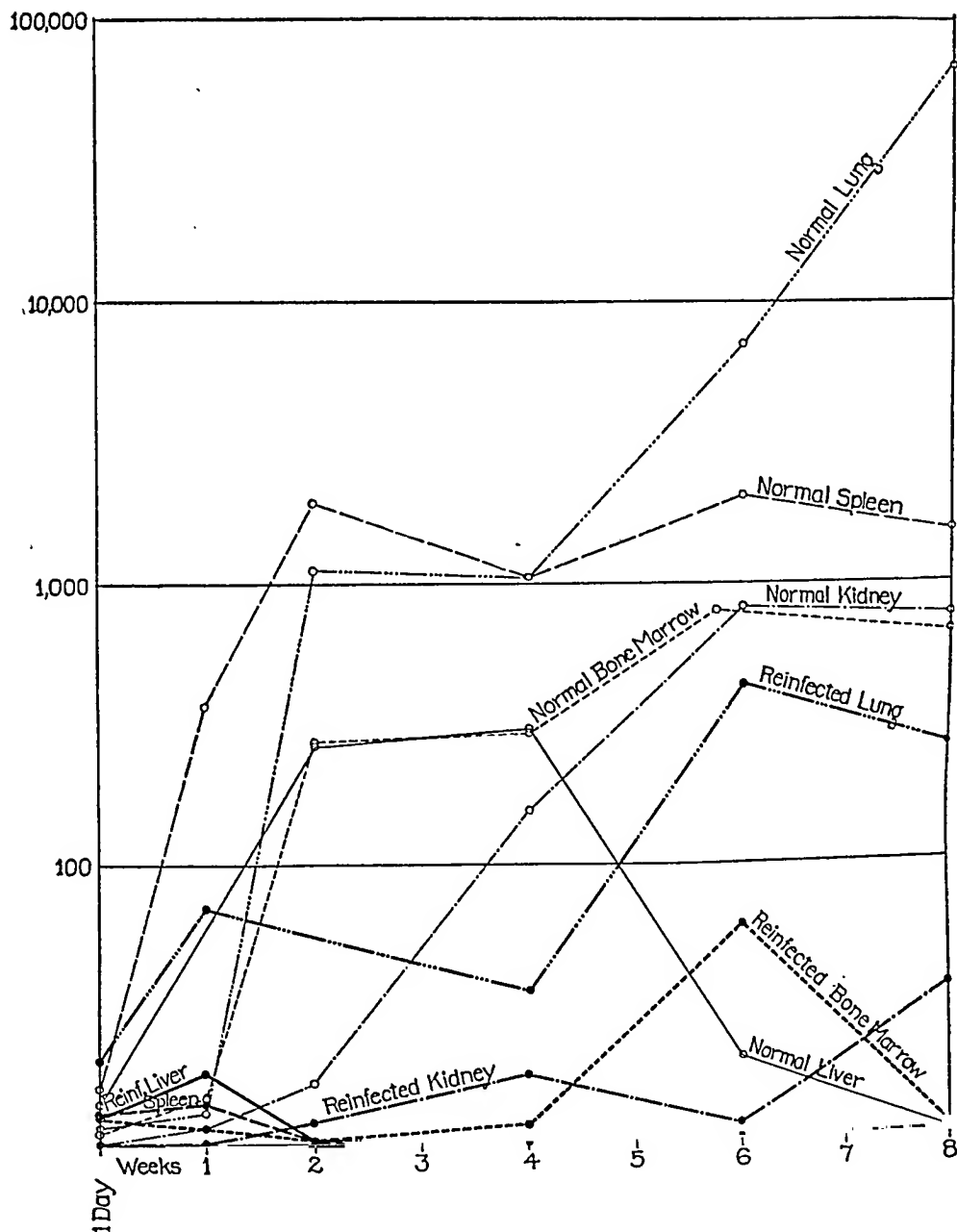
At intervals of 1 day, 1, 2, 4 and 6 weeks and 2 months equal amounts by weight of suspensions of ground lung, liver, spleen, kidney and bone marrow in varying dilutions were seeded directly upon tubes, three of Dorset's and three of Petroff's egg media, and similarly after sodium hydroxide treatment. Two or three rabbits were used for each interval. The number of colonies of tubercle bacilli appearing on the surface of each tube was repeatedly determined, the final reading being

made at the end of 3 months. Sections of all the organs were taken for microscopic examination from the immediate neighborhood of those parts that were cultured. In the reinfected animals care was observed to avoid culturing tissues that were apparently affected by the primary infection. In addition sections of the primary



TEXT-FIG. 1. Each point plotted on the light lines represents the average number of colonies cultured from 10 mg. of a given organ from each of two or three rabbits at varying intervals of time following a primary intravenous inoculation of 0.01 mg. of tubercle bacilli of human type (P 48 A). The points plotted on the heavy lines represent the average number of colonies cultured from the same amount of tissue of a similar number of rabbits reinfected with 0.01 mg. of the same strain 6 months after a primary intravenous inoculation of 0.001 mg. of P 48 A per kilo.

lesion were also prepared for microscopic study. The sections were stained with hematoxylin and eosin and by the Ziehl-Neelsen method for tubercle bacilli with hematoxylin as a counterstain.



TEXT-FIG. 2. Each point plotted on the light lines represents the average number of colonies cultured from 10 mg. of a given organ from each of two or three rabbits at varying intervals of time following a primary intravenous inoculation of 0.01 mg. of tubercle bacilli of bovine type (Bovine C). The points plotted on the heavy lines represent the average number of colonies cultured from the same amount of tissue of a similar number of rabbits reinfected with 0.01 mg. of the same strain 6 months after a primary intravenous inoculation of 0.001 mg. of tubercle bacilli of human type (P 48 A) per kilo.

The fate of the human and of the bovine type tubercle bacilli in the organs of the normal and reinfected animals has been reported in detail (6, 1929) and is contrasted in Text-figs. 1 and 2. Each point plotted represents the average of two or three rabbits except when the cultural characteristics of the bacilli and the associated histological changes were definitely due to residual primary lesions. This necessitated the exclusion from the curves of 7 out of 90 observations of the liver, spleen and bone marrow of rabbits reinfected with human or bovine bacilli. The inclusion of these data would not materially change the trend of these curves. In the lung and kidney however, the persistence of the primary lesion necessitated the omission of 20 out of 60 observations. In the lung, moreover, there were sometimes lesions that could not be clearly ascribed either to the primary infection or to the reinfection. This explains the irregularity of the curves for this organ. In comparing the heavy lines indicating reinfection with the light lines indicating primary infection the immediate destruction of the bacilli with the former is obvious.

The fate of these bacilli in the 26 normal and in the 30 reinfected rabbits is here correlated with a study of the associated lesions. This relation as observed in the liver, spleen and bone marrow is described first, because residual lesions and tubercle bacilli of first infection were very rarely found in these organs. The terminology used in the description of the various cells observed is discussed in a former report (5).

The Liver, Spleen and Bone Marrow after Reinfection with Human and Bovine Type Tubercle Bacilli

Findings after 24 Hours.—

Reinfection with Human Strain.—In Rabbit R 14¹ there was an extensive pulmonary tuberculosis remaining from the primary infection; 720 colonies were isolated from the lung. 3 colonies were obtained from the liver. There was a marked accumulation of mononuclear leucocytes in the liver sinusoids; many of these were detached Kupffer cells. Polymorphonuclears were very rare amongst them. At the periphery of the lobule the mononuclears were often gathered into compact nodules spreading the liver cords apart. Some of them had abundant reticulated cytoplasm. They contained no tubercle bacilli or acid-fast particles. Essentially the same observations were made in Rabbit R 16 (Fig. 3).

In the lung of Rabbit R 15 two isolated tubercles remained from the primary infection and 56 colonies were isolated from this organ. 12 colonies were cultured from the liver. There were fewer mononuclears and more polymorphonuclears than in the previous rabbit. Occasionally there were diffuse infiltrations of the liver sinusoids localized at the periphery of the lobule. The cytoplasm of the mononuclears was less abundant (Fig. 2).

¹ The numbers used to designate the animals are the same as in the paper on the fate of the bacilli of reinfection (6, 1929).

Reinfection with Bovine Strain.—A similar difference was found between two rabbits, Nos. R 42 and R 43, reinfected with bovine type bacilli. In one, No. R 43, with only a few residual lesions in the lung, from which 20 colonies were cultured, the liver showed a diffuse infiltration of the sinusoids by polymorphonuclear and mononuclear cells. In No. R 42, the lung of which was affected by a massive caseous tuberculosis with excavation, and yielded 8,300 colonies, the liver sinusoids were free of infiltrating cells and the mononuclear nodules were smaller, less numerous, more sharply circumscribed, had more abundant cytoplasm and much less prominent polymorphonuclear intermixture than in No. R 43. In No. R 43 the polymorphonuclears were more often free and intact; in No. R 42 they were more often engulfed by the mononuclears and disintegrating within them. 11 colonies were isolated from the liver of the former rabbit; no tubercle bacilli could be cultured from the latter.

Primary Infection with Human Strain.—From the livers of Nos. R 1 and R 2, 14 and 18 colonies respectively were isolated. There was a slight diffuse infiltration of the liver sinusoids by polymorphonuclears. Many of the Kupffer cells were swollen and their nuclei vesicular, and in the cytoplasm were occasionally seen coarse deeply staining acid-fast globules. Some of these cells were detached and rarely small groups of them were found in the sinusoids (Fig. 1).

Primary Infection with Bovine Strain.—In Rabbits R 29 and R 30 essentially the same though more intense reaction was found in the liver; 13 colonies were isolated from the first and 3 from the second rabbit.

From these findings it is clear that the immediate inflammatory reaction as expressed by the outpouring of polymorphonuclears and the accumulation of mononuclears is both intensified and accelerated in the reinfected animal. As a rule fewer bacilli are isolated after reinfection than after primary infection, indicating a greater initial destruction of the bacilli. This initial destruction is most complete in the presence of extensive residual primary lesions. It is associated with sharply circumscribed nodular accumulations of mononuclears in which the polymorphonuclears remain only as infrequent disintegrating cells within the abundant cytoplasm of the mononuclears; there is no general infiltration of the tissues by these cells. In the presence of slight or healed primary lesions the inflammatory reaction is more generalized and extensive, the nodular accumulation of mononuclears is less sharply demarcated, the polymorphonuclears are much more abundant and their phagocytosis by mononuclears, whose cytoplasm is slightly developed, is less in evidence; the bacilli are less effectively destroyed.

Findings after 1 Week.—

Reinfection with Human Strain.—From the liver of Rabbit 17 no colonies were obtained; from No. R 18 one single colony was cultured; both rabbits harbored residual primary lesions in the lung. In both, the lesion in the liver was inconspicuous and consisted of a few minute epithelioid and giant cell tubercles up to 150 micra in diameter. Isolated Langhans giant cells were also found in the sinusoids.

There was no primary lesion in the lung of No. R 19. 8 colonies were isolated from the liver; the tubercles were much more numerous and larger than in the previous rabbit, and isolated giant cells were less frequent. Some of the tubercles were resolving.

Reinfection with Bovine Strain.—There was a slight residual primary lesion in the lung of No. R 45. 31 colonies of bovine type were isolated from the liver, indicating considerable multiplication of the microorganism. The associated lesion was extensive, consisting of larger and more numerous compact tubercles than in any animal thus far described. Giant cells were less frequently found than in the other rabbits of this interval, and surrounding some of the epithelioid cells were mononuclears, amongst which mitotic figures were occasionally seen. A single tubercle bacillus was found within an epithelioid cell (Fig. 4). Rabbit R 44 is discussed below.

Primary Infection with Human Strain.—38 colonies were isolated from the liver of No. R 4. In the sinusoids minute collections of polymorphonuclears were occasionally seen intermixed with several large mononuclears with vesicular nuclei, in the cytoplasm of which acid-fast particles or a rare intact tubercle bacillus was sometimes found. Mononuclears were more numerous in similar collections in the liver of No. R 3; 11 colonies were isolated.

Primary Infection with Bovine Strain.—Essentially the same histological changes were found in Rabbits R 31 and R 32. Fewer colonies were isolated.

Thus again, 1 week after inoculation the reaction in the tuberculous animal is much further advanced than in the normal animal. With the first infection there are only minute collections of polymorphonuclears and mononuclears; whereas after the reinfection well formed mature epithelioid and giant cell tubercles have appeared. The bacilli have entirely disappeared from that reinfected rabbit in which, in association with the persistence of the primary lesion, tubercles are very inconspicuous, and have been less completely destroyed in that animal in which, in association with the complete disappearance of the primary lesion, tubercle formation is more widespread.

Bacilli of the more virulent type had actually multiplied in one animal. This multiplication was associated with a more extensive lesion in which some of the tubercles were still in an early stage of develop-

ment with continued mitosis of mononuclears, although some were mature and some already resolving.

Findings after 2 Weeks.—

At the end of this period only occasionally was a single colony or several colonies of bacilli, whether of human or bovine strain, isolated from the liver of 19 of the 20 reinfected rabbits. The single exception was Rabbit R 49 sacrificed 4 weeks after reinfection with bovine type bacilli. From the liver of this rabbit 18 colonies were isolated and evidence was obtained of invasion of the blood stream by the bacilli from the primary lesion as in Rabbit R 44 described below. Together with the disappearance of the bacilli of reinfection the lesions had retrogressed, leaving only an occasional Langhans giant cell or a collection of disintegrating epithelioid cells. These were characteristically found in the lymph vessels in the periportal spaces, which were often filled with vacuoles (Fig. 6), and there were more of them in rabbits reinfected with the bovine type, although the bacilli were just as rarely isolated from these animals.

Normal rabbits similarly infected showed marked multiplication of the bacillus. Thus 380 colonies were isolated from Rabbit 34, 2 weeks after a bovine infection. There were numerous oval tubercles composed of a core of epithelioid cells and occasionally giant cells surrounded by large mononuclears, often in mitosis (Fig. 5). Intact tubercle bacilli and acid-fast particles were found within the epithelioid cells and the first stages of caseation had appeared in some of the tubercles. Similar observations were made with the human primary infection except that caseation was not noted.

Two weeks after inoculation both the bacilli and the associated lesion in the liver of the reinfected animal have largely disappeared, whereas in the normal animal the bacilli are rapidly multiplying and the lesions rapidly extending.

The bacilli of reinfection are not completely destroyed in the liver even 2 months after reinfection, when an occasional isolated colony may be cultured. The connective tissue cells in the periportal canals are much increased, consisting of lymphocytes, occasional plasma cells, mononuclears and fibroblasts. There still may be found a rare giant cell, as after 2 weeks.

The relations between the fate of the bacilli of reinfection and the histological changes in the spleen and bone marrow were the same as in the liver. From these organs isolated colonies were repeatedly cultured in the absence of any microscopic tuberculous lesions.

The Lung after Reinfection with Human and Bovine Type Tubercle Bacilli

The correlation between the fate of the bacilli and the histological changes in the lung could not always be ascertained with the same degree of certainty as in the liver, spleen and bone marrow because a lesion of variable character often remained from the primary infection in this organ, and the number of contained bacilli varied accordingly. Furthermore, as will be shown later, in some rabbits there was clear evidence of tubular spread of the lesion from these foci. The age of a given lesion as indicated by its appearance could therefore not always be used as a criterion to differentiate between that due to endogenous spread and that due to the exogenous reinfection. Nevertheless certain definite relations were observed that in general are the same as those in the other organs.

Findings after 24 Hours.—

Reinfection with Human Strain.—Two isolated tubercles remained from the primary infection in No. R 15. 56 colonies were obtained in cultures. There were localized, though not sharply demarcated, infiltrations of the alveolar septa by large mononuclears with some polymorphonuclears. Macrophages laden with carbon particles often occupied what was left of the alveolar spaces. There was a considerable general accumulation of polymorphonuclears in the small vessels and in the septa. Both phases of the reaction were more marked than at the same interval with the primary infection.

There were small numbers of discrete tubercles in the lung of No. R 16. 1,030 colonies were isolated. The nodal thickenings were more extensive and more sharply delimited than in No. R 15. There were very few polymorphonuclears. The primary lesion in No. R 14 was extensive; 720 colonies were isolated. Histological changes due to reinfection could not be determined.

Reinfection with Bovine Strain.—There were a few residual tubercles in the lung of No. R 43, some with calcification. 20 colonies were isolated. There was intense acute inflammation and an accumulation of polymorphonuclears. These often plugged small blood vessels and infiltrated the septa in compact masses. Here and there were found small nodal collections of mononuclears intermixed with polymorphonuclears. No. R 42 showed a very extensive primary lesion. 8,300 colonies were isolated. It could not be ascertained if there was any lesion due to reinfection.

Thus the difference in reaction between the normal and the tuberculous animal as observed in the lung after 24 hours is the same as in the

liver, but more intense. Alveolar phagocytes play a more important rôle in the reaction of the reinfected than of the normal animal.

Findings after 1 Week.—

Reinfection with Human Strain.—One isolated tubercle 4 mm. in diameter remained in the lung of No. R 17. 6 colonies were cultured from an area that grossly appeared unaffected, but microscopically showed rare nodules up to 320 micra in diameter. These were often situated in alveolar spaces. They were composed of a core of young and mature epithelioid cells, or a rare giant cell, often containing carbon particles and frequently surrounded by a ring of mononuclears, polymorphonuclears and lymphocytes (Fig. 8). Some nodules were less mature and mitoses of mononuclears were seen about them. No. R 18 showed smaller and more mature tubercles probably due to reinfection. There were cavities in the base of both lungs. 61 colonies were isolated.

No lesions were visible in the gross in the lung of No. R 19. 40 colonies, apparently due to the reinfection, were isolated. The tubercles were from four to six times more numerous than in Rabbit R 17 and were considerably larger, a few as large as 500 micra. The accumulation of mononuclears about tubercles was much more conspicuous and diffuse, and mitotic figures were more frequent. Some of the larger tubercles showed the first stages of caseation (Fig. 9). The lymph follicles were not affected. Tubercle bacilli could not be seen histologically.

Reinfection with Bovine Strain.—There were two residual tubercles in each lung of No. R 45. 70 colonies were isolated. The lesion was essentially the same as in No. R 19 but more extensive. Here too caseation was found in a larger tubercle. The lymph follicles remained intact. No tubercle bacilli were seen. There remained very extensive primary lesions in the lung of No. R 44; 30,000 colonies were isolated. No lesions due to reinfection could be distinguished.

Primary Infection with Human Strain.—48 and 50 colonies respectively were isolated from the lung of Nos. R 3 and R 4. There were very rare nodular collections of mononuclears with hyperchromatic nuclei, with occasional mitosis at the periphery. Within these nodules were one or two cells with abundant cytoplasm and vesicular nuclei, sometimes containing two or three tubercle bacilli (Fig. 7). The lymph follicles were hypertrophied, showing a moderate number of mitotic figures. Occasionally they contained several aggregations of large cells, in which acid-fast particles were found.

Primary Infection with Bovine Strain.—The mononuclear nodules were larger and more frequent in Rabbits R 31 and R 37. Polymorphonuclears persisted in them. Bacilli were found within the mononuclears. No direct culture was obtained.

One week after a first inoculation there are minute interstitial nodules of mononuclears in the lung. After reinfection tubercles are formed; they are frequently situated in the alveolar spaces, and the

epithelioid cells often contain carbon particles. The intrapulmonary lymph follicles in the reinfecting animal are not affected; in the primarily infected animal they are hypertrophied and contain early tuberculous foci.

As in the other organs the lesion is less extensive and more mature in the animal in which, in association with the persistence of the primary lesion, the bacilli of reinfection are almost completely destroyed. They are more extensive and in an earlier stage of development with continued mitoses of mononuclears in the animal in which, in association with the complete or almost complete healing or disappearance of the primary lesion, the bacilli of reinfection, whether of human or bovine type, are less completely destroyed.

It is noteworthy that in this latter group, small foci of caseation were seen in tubercles 1 week after reinfection. With the primary infection caseation was not found until the 2nd week with the bovine type, and not until the 4th week with the human type bacilli.

Findings after 2 Weeks.—

The tubercles were resolving. The cytoplasm of the epithelioid cells was filled with vacuoles, which in some coalesced, leaving only shreds of cytoplasm. The dissolution of the epithelioid cells had caused wide spacing between them. Lymphocytes, fibroblasts and capillaries surrounded and permeated the tubercles. Only a very small number of bacilli could be cultured; 6 colonies were isolated from one rabbit reinfecting with the human type bacillus. With the bovine reinfection the disintegration of epithelioid cells was similar but much less in degree (Fig. 12). On the other hand the lesion was rapidly advancing both with human and bovine type primary infections. Early tubercles composed of a core of young epithelioid cells containing numerous bacilli surrounded by a ring of mononuclear cells, many in mitosis, with islands of young epithelioid cells in the lymph follicles, characterized the lesion of the primary infection with the human type bacillus. 1,580 colonies were isolated from such a lung. The bovine type primary infection at this time showed in addition an extensive diffuse infiltration of mononuclears in the septa with numerous mitoses, and the first stages of caseation had appeared in the larger tuberculous foci where numerous tubercle bacilli were found (Fig. 11). 1,200 colonies were isolated from such a lung.

Findings after 4 Weeks.—

4 weeks after reinfection the bacilli both of the human and bovine types had largely disappeared. Except for a rare very much disintegrated tubercle composed

of phantom-like vacuolated epithelioid cells, the lung after reinfection with the human type was free from lesions due to exogenous reinfection. 10 and 20 colonies respectively were isolated from Rabbits R 22 and R 24. From Rabbits 48 and 50, reinfected with the bovine bacillus, 10 and 60 colonies respectively were isolated. The residual tubercles were more numerous and their disintegration less far advanced. Frequently microscopic tissue defects had resulted from the disintegration of the epithelioid cells.

Two weeks after reinfection resolution of tubercles in the lung is beginning with the human type, and with both types the bacilli have largely disappeared. With the bovine type this process of disintegration is not conspicuous until the 4th week.

Although this is usually the fate of the bacilli of reinfection and the lesions to which they give rise in the lung, occasionally considerable multiplication of the bovine bacillus is found in this organ. This is illustrated in Rabbit R 53.

X-ray examination of the lungs before reinfection showed no tuberculous lesions. When the rabbit was killed 6 weeks after reinfection there were uniformly distributed discrete tubercles 1 to 3 mm. in diameter, some with punctate caseous centers. 540 colonies with the dysgonic characteristics of the bovine type were isolated. Microscopically the lesions were of two types. In one interstitial tubercles were surrounded by a heavy ring of lymphocytes, which together with ingrowing capillaries permeated the tubercles (Fig. 14). There were giant cells at the periphery. There were no tubercle bacilli and there was no caseation in such tubercles. Some of them were resolving. The larger and less frequent lesion was of combined interstitial and intraalveolar nature. Very rarely an isolated tubercle bacillus was seen in the neighborhood of minute foci of caseation. Infrequent mitotic figures were observed amongst the mononuclears infiltrating the septa about the pneumonic foci. Here there were many giant cells. By far the greater part of the parenchyma was free from tuberculosis. The lymph follicles were unaffected.

By contrast in a normal animal similarly infected most of the parenchyma was consolidated by conglomerate tubercles with extensive foci of caseation. Caseous pneumonia predominated, and the advancing edge of the focus was a mass of mononuclear cells with frequent mitoses (Fig. 13). Tubercle bacilli were found throughout the lesion but were especially numerous in the pneumonic areas. Lymphocytes did not surround or infiltrate the tubercles to any extent. The intrapulmonary lymph follicles were largely replaced by tuberculous tissue with central foci of caseation. 12,500 colonies were isolated from this lung.

It is evident that with the complete disappearance of the primary lesion bovine bacilli may persist in moderate numbers in the lung even 6 weeks after reinfection in small localized pneumonic areas with slight foci of caseation about which mitosis of mononuclears has not

yet ceased, although they have completely disappeared from the small, discrete, healing (Fig. 14) and resolving interstitial tubercles in the same lung. The intrapulmonary lymph follicles were not affected. In a primary infection at this time there is a massive multiplication of the microorganism in association with a generalized pneumonic process with unabated multiplication of mononuclears, extensive caseation and destruction of the intrapulmonary lymph nodes. It is noteworthy that even in Rabbit R 53 the bacilli of reinfection have completely disappeared from the liver and spleen and are present in much smaller numbers in the bone marrow than in the lung. This difference, as well as the larger initial lesions, and the slower resolution of these in the lung than in the other organs even in animals that completely destroyed the bacilli of reinfection, indicates that the acquired immunity is superimposed on the natural resistance of a given organ and is greater in an organ that has an initial greater resistance against the tubercle bacillus.

The Occurrence in the Kidney

Few tubercle bacilli of reinfection were isolated from the kidney after reinoculation with the human or bovine types. No inflammatory reaction was noted 24 hours after intravenous infection and as a rule no tubercles formed subsequently. That some tubercle bacilli of reinfection reached the kidney was indicated by the few residual bacilli isolated from rabbits in which the primary lesions had disappeared, in association with a rare abortive tubercle. Such instances were more frequent in the bovine type series.

These observations suggest that in the presence of a certain degree of immunity the small numbers of tubercle bacilli that are retained in the kidney are destroyed by the mononuclear cells *in situ* without causing any inflammation and without subsequent tubercle formation. Yet in the normal animal the kidney is the organ where the bacilli reach great numbers and where they cause extensive lesions, second only to those in the lung.

The Relation of Residual Primary Lesions to Reinfection

It is evident therefore from the foregoing experiments that in all the organs the bacilli of exogenous reinfection and the abortive lesions to which they give rise soon disappear whether they are of the identical

strain that was used for the primary infection or another, even if of more virulent type. Furthermore, the more extensive the primary lesion the more rapid and complete the destruction of these bacilli and the less conspicuous and more ephemeral the lesions caused by them. Yet these residual lesions in the lung and kidney themselves may harbor innumerable bacilli of the first infection, and may gradually extend until the whole organ is almost completely destroyed. A correlation between histological changes and the fate of bacilli in these animals indicates that there is no incongruity between this demonstrated immunity to the more virulent bovine bacillus of reinfection and the persistence and continued multiplication of the less virulent human type bacilli in the residual primary lesions. This is illustrated by Rabbit 44.

X-ray examination of the lung before reinfection with bovine type bacilli showed an extensive disseminated tuberculosis. 1 week after reinfection the animal was killed and 30,000 colonies of eugonic (presumably human) type were isolated from the relatively unaffected part of the lung. In the gross there was an extensive pulmonary tuberculosis with pus-containing cavities, and tuberculosis of the joints of all four extremities. Microscopically, the greater part of the lung parenchyma was consolidated by conglomerate foci of compact, homogeneous and cell-free caseous tissue in which tubercle bacilli were found with great difficulty. Here and there however there were caseous foci infiltrated with large numbers of both mononuclear and polymorphonuclear cells; the caseous material was broken up and often appeared as islands in a mass of pyknotic dead cells. Here tremendous numbers of bacilli, at times in actual colonies, had accumulated, apparently multiplying in the softened caseous material (Fig. 15). The surrounding partially thrombosed blood vessels occasionally showed tubercle bacilli. Some of these foci had broken into the bronchi (Fig. 16), and aspirated caseous material carrying large numbers of bacilli were seen lying free in the alveoli in the relatively unaffected parts of the lung from which the culture was made. Fresh foci of caseation surrounded by epithelioid cells in which there were bacilli in smaller numbers were also found in this location.

From the liver, spleen and bone marrow 28, 69, and 12 colonies respectively, all of the eugonic type, were isolated. Polymorphonuclears were conspicuous in these organs. Deeply staining acid-fast particles and hemoglobin pigment were found in the phagocytic mononuclears of these organs, which cells were at times seen in mitotic division. Rare minute epithelioid and giant cell tubercles were seen in them (Fig. 10).

From the kidney 1,630 colonies of the same type were isolated. There was a large wedge-shaped lesion undergoing a process of softening similar to that in the lung.

Similar observations were made in other rabbits of this series.

Thus the caseous foci in lesions of first infection undergo softening in a certain stage of their development. In these areas there are invading mononuclear and polymorphonuclear cells, which may undergo necrosis. Here there seems to be rapid and unhindered multiplication of the bacillus. Disintegrating caseous foci rupture into the bronchi and tremendous numbers of living tubercle bacilli may be aspirated into alveoli far removed from the original focus, there setting up fresh lesions, which in turn may undergo the same cycle and by tubular spread gradually cause the destruction of the organ. A similar process may take place in the kidney.

As softening progresses blood vessels may be involved in the rapidly extending caseous process, and although thrombosis occurs in most of them bacilli may invade the blood stream, and may be distributed to the various organs as with primary intravenous inoculation. In the liver they induce an acute inflammatory reaction and many of them are destroyed by the Kupffer cells. These cells undergo mitosis and the remaining bacilli are destroyed by abortive tubercles as were the bacilli of exogenous reinfection.

It is noteworthy that the tubercles were much more numerous and larger, and that there was no acute inflammation in the liver of No. R 45 (Fig. 4), a rabbit re-infected similarly to No. R 44 with bovine type bacilli. There were slight, healing residual lesions in the lung and 31 colonies of the bovine type were isolated from the liver. In this rabbit therefore the bacilli are due to the exogenous reinfection of the previous week. In No. R 44 however (Fig. 10), the infrequency of well formed tubercle in the liver suggests that the bacilli of exogenous reinfection of the previous week were destroyed and left little if any trace of their presence, whereas the abundance of polymorphonuclear leucocytes and the cultural characteristics of the bacilli indicate that they are more recently derived from the pulmonary lesion of primary infection.

That the bacilli that invade the various organs from the blood stream are there destroyed was clearly seen in Rabbit R 16. Here 24 hours after reinfection with human type tubercle bacilli only three colonies were cultured from the liver. No tubercle bacilli were obtained from the spleen. Nevertheless these organs contained several resolving epithelioid and giant cell tubercles, which must be attributed to the presence of tubercle bacilli at an earlier period brought there by the blood from the primary lesion, which showed areas of softening.

It is plain that the moderate numbers of tubercle bacilli of first infection that reach the various organs by way of the blood stream are rapidly destroyed. But in the lung the immunity brought about by

the first infection is overwhelmed by the massive seeding of bacilli from the aspirated material from softened areas.

DISCUSSION

From these observations certain inferences may be drawn in regard to the nature of immunity in tuberculosis. On the basis of the demonstration by animal inoculation that tubercle bacilli may persist in the lesions of reinfection Selter (7) has maintained that immunity to tuberculosis is not primarily due to the destruction of the bacilli of reinfection but to the tolerance of the tissue cells to the presence of the bacilli so that they fail to produce tuberculous lesions. Kalbfleisch (3) maintains that reinfected animals do not destroy tubercle bacilli more readily than the normal animal. Hedwal (8) has come to the same conclusion. That tubercle bacilli may persist in the tissues in complete absence of even microscopic lesions has been established by numerous observers. With the cultural method, by which the numbers of bacilli present can be determined, it has been found that a rare isolated tubercle bacillus may remain in tissues in which no lesions are visible with the microscope. However it is clear from the observations reported here that the failure of development of lesions is associated with an all but complete destruction of the bacilli, which may be accomplished within 24 hours in the liver, spleen and bone marrow of the more resistant animals, with moderate quantities of tubercle bacilli of moderate virulence in the presence of extensive primary lesions. With the healing or disappearance of the primary lesion this immediate destruction is less marked and the subsequent destruction of the bacilli is associated with the accelerated appearance of abortive tubercles. The degree of immunity to tuberculosis is therefore a function of the increased capacity of the tissue cells to destroy tubercle bacilli and varies directly with the extent of the primary lesion. However, the immunity is rarely sufficient completely to annihilate the microorganism.

In interpreting the cellular changes that accompany the increased destruction of the bacilli of reinfection one must bear in mind the succession of cells that characterize inflammation in general, namely polymorphonuclears, mononuclears and lymphocytes, and also the conclusion drawn from former experiments (5) that the epithelioid

cell of tuberculosis is a mononuclear cell that has destroyed and partially digested tubercle bacilli or their products.

In the presence of sufficient immunity, such as occurs with the persistence of extensive primary lesions, the bacilli of reinfection may be completely destroyed by phagocytosis in the mononuclear cells *in situ*, and there will be no immediate general or local infiltration of the tissues by polymorphonuclear and mononuclear cells, and no subsequent tubercle formation. This is seen when small numbers of tubercle bacilli of reinfection are retained in the kidney in sufficiently immune animals. When larger numbers reach a given organ, there is a slight accumulation of polymorphonuclears, which the mononuclears encompass and destroy, together with the bacilli, within 24 hours. Most of the nodules progress no further and by the end of a week they have all disappeared. A rare, minute, mature epithelioid and giant cell tubercle may be left indicating that in this focus the disintegration of the tubercle bacilli was incomplete. After this time the few residual tubercles resolve and the tissues return to their normal condition.

In the presence of partial immunity, such as occurs with the complete or almost complete disappearance of the primary lesion, the immediate inflammatory reaction is more intense and generalized, the bacilli are less completely destroyed and the accumulation of both polymorphonuclears and mononuclears continues for a longer time. More of the mononuclear nodules progress to tubercle formation at the end of a week and if the growth of the bacillus is incompletely suppressed new mononuclear cells accumulate and continue to form by mitosis around these tubercles. Thus the destruction of the bacilli of reinfection is a function of the acceleration and intensification of the inflammatory process. The more complete destruction is associated however not with the greater intensification but with the greater acceleration of the sharply localized inflammation. It has been seen that small numbers of bacilli can be destroyed by the mononuclear phagocytes *in situ* without inflammation. Hence the greatest immunity is associated with the absence of any lesion, a partial immunity with the more rapid formation of tubercle followed by its rapid disappearance.

Caseation developed in the lung of rabbits with little or no primary lesion 1 week after reinfection with either the human or bovine type bacillus in association with slight multiplication of the microorganism.

In the lung of normal rabbits 2 weeks after infection with the human type bacillus no caseation developed despite the presence of very much larger numbers of bacilli. The earlier appearance of caseation in the reinfected animal can be explained in part at least by the well known fact that the tubercle bacillus and its products destroy the cells of the tuberculous animal but leave unharmed the tissues of the normal animal (8).

It is noteworthy that the intrapulmonary lymph follicles remained intact in the reinfected animal even in the presence of the smallest degree of immunity, whereas in the normal animal they became the seat of extensive tuberculosis. This would indicate that most of the bacilli introduced intravenously into rabbits do not reach the lymph follicles primarily as maintained by Krause, but are drained from foci in the lung parenchyma, as recently maintained by Vorwald (10). In the reinfected animal they are destroyed wherever they localize and few if any are carried to the tributary lymph nodes.

The persistence of tremendous numbers of human type tubercle bacilli in the primary lesions of some reinfected rabbits and their gradual extension to the ultimate almost complete destruction of the organ despite the demonstrated immunity to the more virulent bovine bacilli of exogenous reinfection, are due to the process of softening and cavity formation that overtakes the old caseous foci. There is a massive accumulation of viable bacilli in the unorganized material of the softened areas, which leads to a rapid extension of the caseation and to tubular spread of tremendous numbers of bacilli (Fig. 16), which overwhelm the existing immunity. By the same process these bacilli enter the blood vessels but owing to the rapid formation of thrombi only moderately small numbers invade the general circulation whence they are filtered and destroyed by the organs, like the bacilli of exogenous reinfection (Fig. 10).

CONCLUSIONS

1. Immunity to reinfection is a function of the increased capacity of the mononuclear phagocytes to destroy tubercle bacilli and varies directly with the extent of the primary lesion; however it is rarely sufficient to annihilate completely the microorganism. This acquired immunity is superimposed on the natural resistance of a given organ.

2. In the presence of sufficient immunity, such as occurs with the persistence of an extensive primary lesion, small numbers of tubercle bacilli are destroyed by the mononuclear cells *in situ* without local or general infiltration of the tissues by polymorphonuclear or mononuclear leucocytes. Larger numbers of bacilli are destroyed within 24 hours by an accelerated formation of sharply localized nodules of mononuclear phagocytes. These progress no further and are absorbed or result in inconspicuous microscopic collections of epithelioid and giant cells.

3. In the presence of less immunity, such as occurs when the primary lesion has almost completely healed, the immediate inflammatory reaction is more intense and diffuse and persists longer. It results in a less rapid disappearance of the bacilli and in a more extensive formation of tubercles. These appear much earlier than in the normal animal and soon resolve.

4. Tubercle bacilli of reinfection may be destroyed even though the primary lesion in the lung and kidney is progressive. This is due to an unhindered extracellular multiplication of the bacilli in the caseous foci that undergo softening and excavation. Resistance may be overwhelmed by the spread of tremendous numbers of living bacilli from these foci through the bronchi or renal tubules, while the moderate numbers of reinfecting bacilli reaching the organs by way of the blood stream are destroyed.

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EXPLANATION OF PLATES²

All microphotographs were made from sections stained by hematoxylin and eosin except those shown in Figs. 6, 8, 10, 15 and 16, which were stained by the Ziehl-Neelsen method and counterstained with hematoxylin.

PLATE 10

FIG. 1. Liver of Rabbit 2, 24 hours after a primary infection with tubercle bacilli, human type. 18 colonies were isolated. Swollen Kupffer cells and slight infiltration of the sinusoids with polymorphonuclears. $\times 600$.

FIG. 2. Liver of Rabbit 15, 24 hours after reinfection with tubercle bacilli, human type. 12 colonies were isolated. Slight residual primary lesions in the lung. Diffuse infiltration of liver sinusoids with polymorphonuclear and mononuclear cells, the latter with scanty cytoplasm. $\times 600$.

FIG. 3. Liver of Rabbit 16, 24 hours after reinfection with tubercle bacilli, human type. 3 colonies were isolated. Moderate primary residual lesions in the lung and kidney. Sharply circumscribed nodule composed of mononuclears with abundant cytoplasm; the polymorphonuclears are almost entirely absent. $\times 600$.

FIG. 4. Liver of Rabbit 45, 1 week after reinfection with tubercle bacilli, bovine type. 31 colonies of the dysgonic type were isolated. Slight residual primary lesion in the lung. Extensive formation of tubercles surrounded by numerous mononuclears. $\times 140$.

PLATE 11

FIG. 5. Liver of Rabbit 34, 2 weeks after a primary infection with tubercle bacilli, bovine type. 380 colonies were isolated. An advancing tubercle with a core of epithelioid cells surrounded by extensive mononuclear infiltration. $\times 200$.

FIG. 6. Liver of Rabbit 47, 2 weeks after reinfection with tubercle bacilli, bovine type. 1 colony in three specimens was isolated. Vacuolated Langhans giant cells in the portal spaces. $\times 200$.

FIG. 7. Lung of Rabbit 4, 1 week after a primary infection with tubercle bacilli, human type. 50 colonies were isolated. A nodule of lymphoid mononuclear cells in a septum; no mature epithelioid cells. $\times 200$.

FIG. 8. Lung of Rabbit 17, 1 week after reinfection with tubercle bacilli, human type. 6 colonies were isolated. Slight residual primary lesion. Mature epithelioid tubercle; no caseation. $\times 200$.

² Approximate magnification is indicated for each figure.

PLATE 12

FIG. 9. Lung of Rabbit 19, 1 week after reinfection with tubercle bacilli, human type. 40 colonies were isolated. No residual primary lesion. Extensive tubercle formation with marked accumulation of mononuclears about the tubercle and caseation in an early stage in the center. $\times 200$.

FIG. 10. Liver of Rabbit 44, 1 week after reinfection with tubercle bacilli, bovine type, 28 colonies of the eugonic type were isolated. Numerous caseous pus-containing cavities in the lung and tuberculosis of the joints. Hemoglobin pigment in a Kupffer cell with mitosis of one of these just below and to the left of the Langhans giant cell; infiltration of sinusoids with mononuclear and polymorphonuclear cells. $\times 600$.

FIG. 11. Lung of Rabbit 34, 2 weeks after a primary infection with tubercle bacilli, bovine type. 1,200 colonies were isolated. Part of a large tubercle with massive accumulation of mononuclears with very few epithelioid cells; caseation in an early stage is seen in the upper portion of the tubercle. $\times 200$.

FIG. 12. Lung of Rabbit 46, 2 weeks after reinfection with tubercle bacilli, bovine type. 300 colonies of the eugonic type were isolated. Regressive intra-alveolar tubercle surrounded and permeated by lymphocytes. $\times 200$.

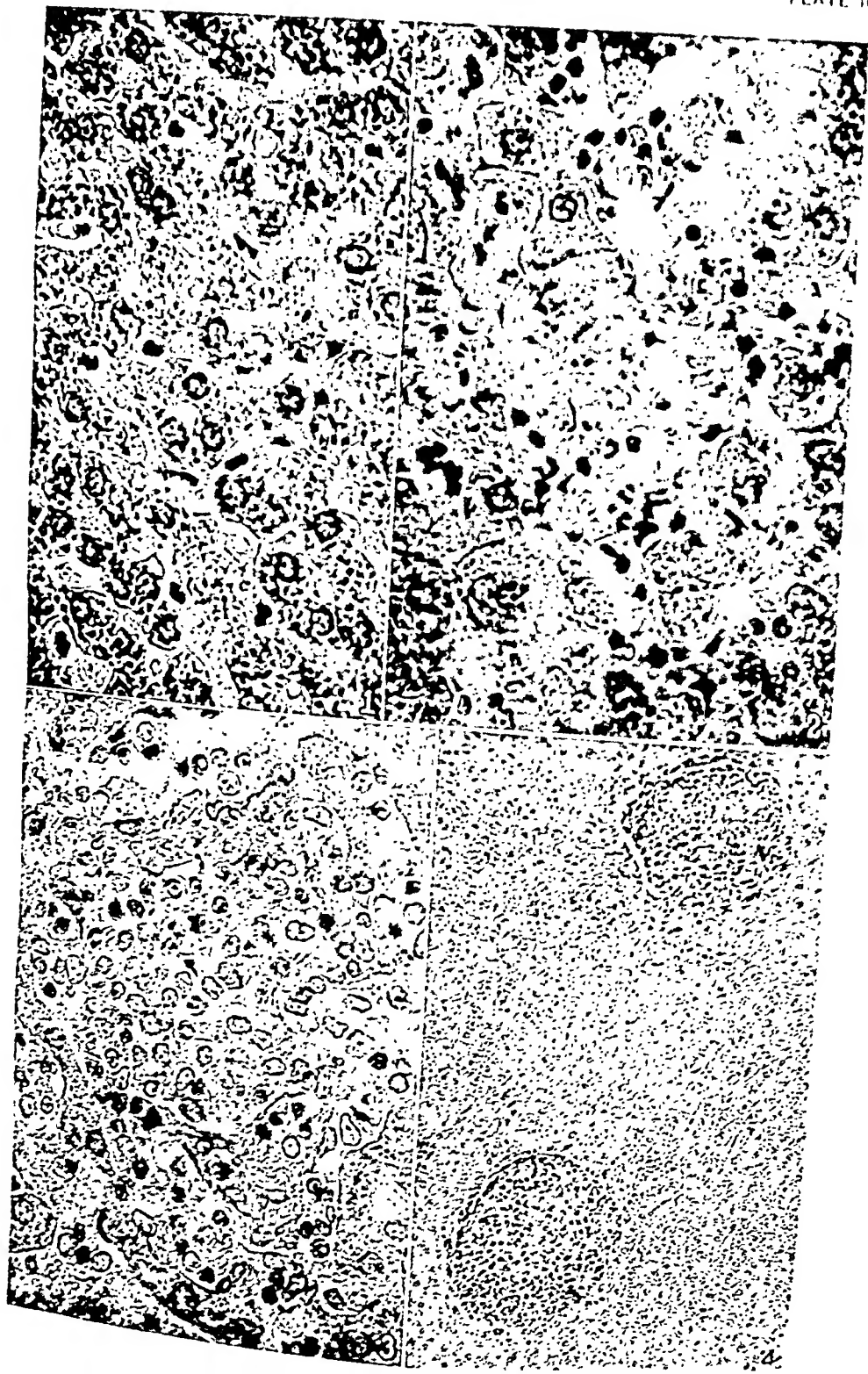
PLATE 13

FIG. 13. Lung of Rabbit 37, 6 weeks after a primary infection with tubercle bacilli, bovine type. 12,000 colonies were isolated. Extensive caseous interstitial tubercles surrounded by numerous mononuclears; caseous pneumonia is prominent. $\times 200$.

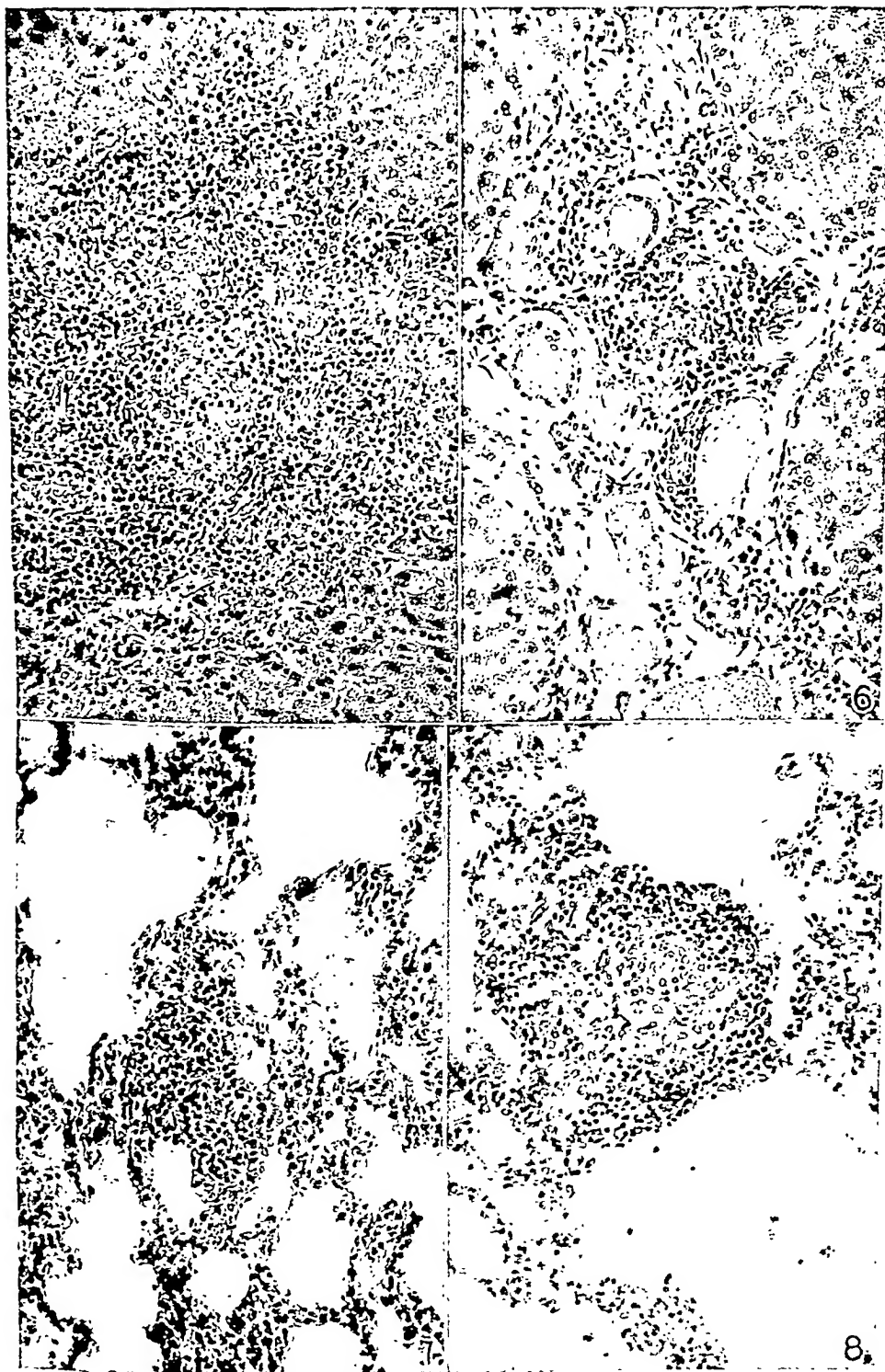
FIG. 14. Lung of Rabbit 53, 6 weeks after reinfection with tubercle bacilli, bovine type. 540 colonies of the dysgonic type were isolated. $\times 200$.

FIG. 15. Lung of Rabbit 44, 1 week after reinfection with tubercle bacilli, bovine type. 30,000 colonies of the eugonic type were isolated. An area of caseous tissue undergoing softening with numerous pyknotic cells and large numbers of tubercle bacilli. $\times 600$.

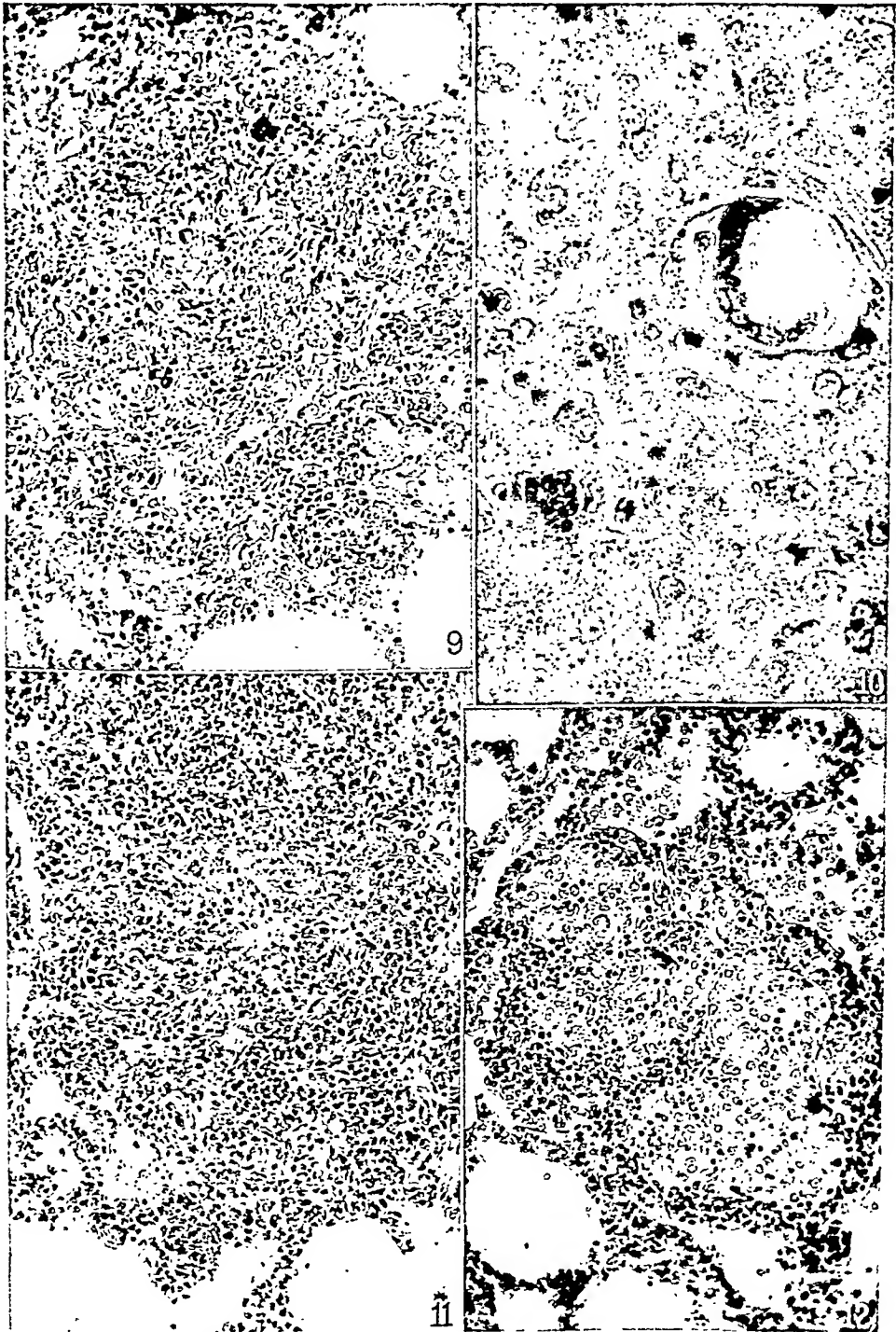
FIG. 16. Disintegrating caseous tissue in a bronchus from the same lung as in Fig. 15. $\times 600$.





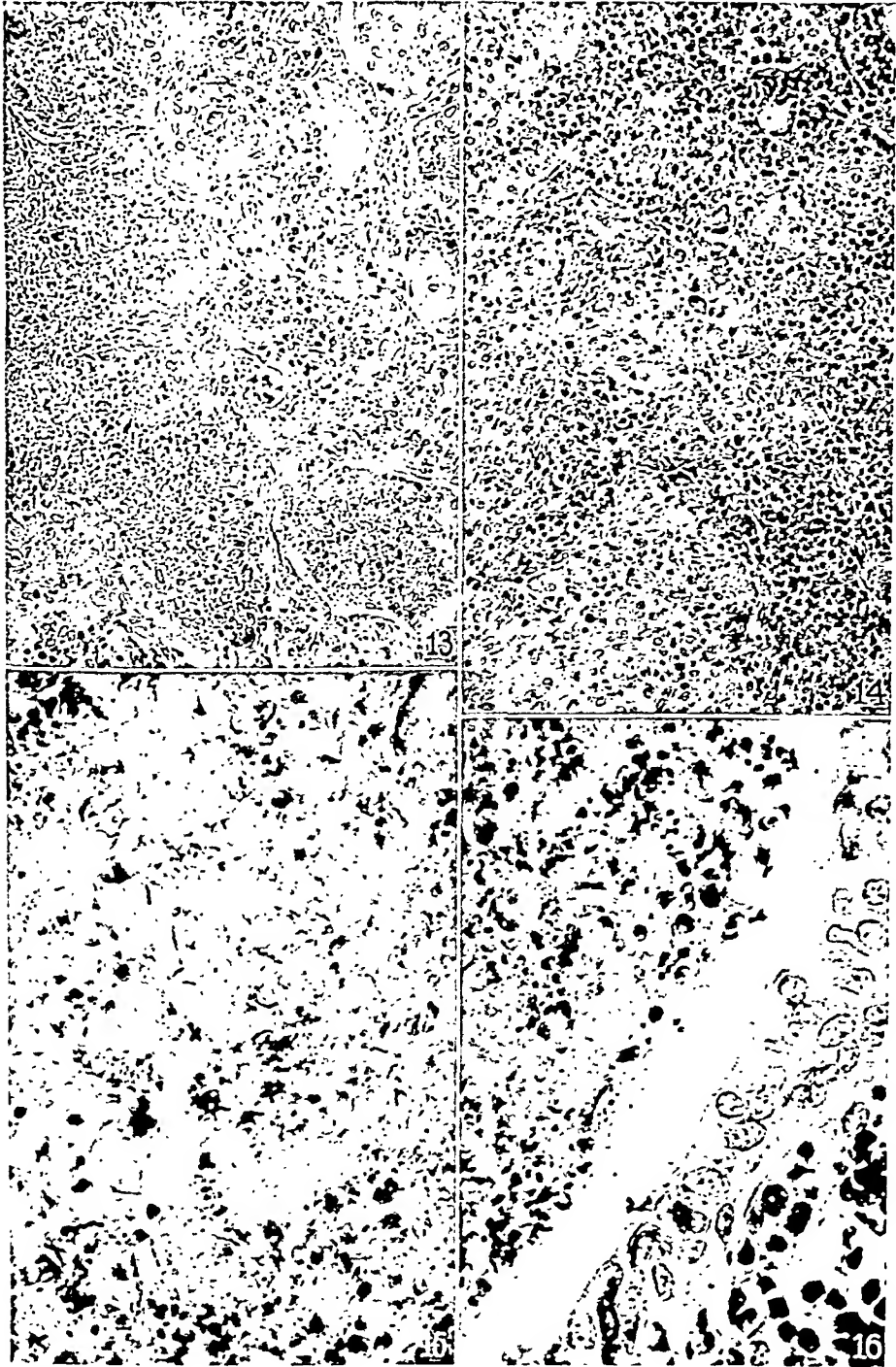


(Lurie: Living tubercle bacilli in organs)



(Lurie: Living tubercle bacilli in organs)





(Lurie. Living tubercle bacilli in organs)

THE EFFECT OF ACIDS AND OTHER SUBSTANCES IN THE PRODUCTION OF ACUTE GASTRIC ULCERS*

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PLATE 14

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Gastric ulcers have been produced by many experimental methods but in very few instances have they shown any tendency toward chronicity. Numerous investigators have attributed to hyperacidity the power of converting acute into chronic ulcers. There are certainly many factors involved but none have been so consistently present as hyperacidity. The fact that a moderate number of ulcers have been recorded in which hyperacidity was not present is evidence enough that other factors play important rôles in the production of the condition. Since the etiology of ulcer is still not well understood we have attempted to study the problem experimentally. It is a problem of the first importance, not alone because of the dangers and disabilities caused by ulcer, but because this so often leads to cancer of the stomach.

O'Shaughnessy (1) recently attempted to produce chronic ulcers in dogs by injecting histamine into the muscular layer of the stomach wall. In two instances of a large series he was able to produce ulcers which resembled the chronic forms seen in man. The same observation had been previously made by Bolton who used gastrototoxic serum and a similar technique. In the remaining experiments of his series, O'Shaughnessy was able only to produce acute ulcers and these healed rapidly. He suggests that histamine is more than likely an important factor in ulcer production. The fact is well recognized that histamine is a powerful stimulant to hydrochloric acid secretion and although subcutaneous injections of histamine do not in themselves produce chronic ulcers, the local injection into the stomach

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wall may, according to some observers, bring this about through an induced over-secretion of acid.

In the experience of Ivy (2) hyperacidity is not of great significance for the chronicity of ulcer. However, many investigators have shown that instillations of hydrochloric acid into the stomach and duodenum produce not only acute and chronic gastritis and duodenitis but also multiple erosions and acute ulcers. Gallagher found that when an element of trauma was added to the administration of acid by mouth, chronic ulcers could be produced.

More recently, the importance of acid in the production of ulcer has been discussed by Lindau and Wulff (3) who have been able to corroborate Buchner's finding that ulcers arise in the areas of activity of gastric juice, whereas the area of production possesses by nature a relatively high power of resistance. Ulcers rarely occur in the area of the fundus glands; they are usually found in the region of the pyloric glands. From their survey Lindau and Wulff conclude, that "in the biochemical theory, we have an exceedingly good explanation of the nature as well as the location of the peptic lesions with ulcer formation, confined to the esophagus, cardia, area of the pyloric glands, duodenum, jejunum (postoperatively) and in Meckel's diverticulum. Everywhere the lesion is to be found in the area of activity of hydrochloric acid."

Ivy and Shapiro (4) in 1925 were able to produce acute ulcers of the stomach in dogs and rabbits on the basis of local anaphylaxis to foreign proteins. In a later series of experiments (5) they describe in detail their method of producing ulcer. They employed egg albumin, beef protein, oat protein, horse serum, edestin, squash and other substances and found that when these substances were injected into the stomach wall without previous sensitization no ulceration resulted, but when previously sensitization had been carried out with the respective sensitizing proteins, ulcerations were found at the site of injection in the stomach. They also noted that the healing times of the ulcers were not affected by the gastric acid. On the other hand, Bolton (6) maintains that the acid of the gastric juice is the chief damaging factor to a devitalized portion of the stomach, and where motor insufficiency occurs, the effect of the acid is more marked and the healing of acute ulcerations is definitely delayed. Although Bolton emphasizes the fact that hyperacidity is concerned as a factor in the pathological process of an ulcer, yet he maintains that proof has never been afforded that an ulcer gives rise to hyperacidity. Dragstedt (7), after producing ulcers in Pavlov pouches in animals by the local injection into the stomach wall, of 4 per cent silver nitrate, concluded that acidity had no effect upon the healing time of acute ulcers.

Methods

In our experiments we tested the effect of injections of various substances into the muscular coat of the stomach and their relation to the production of hyperacidity and acute ulcers.

The following materials were injected: sterile water, normal salt solution, glu-

case 50 per cent, skimmed milk, whole milk, lipo-protein (the commercial preparation, omnadin was used), lipiodol, bismuth subcarbonate, pituitrin S., 5 per cent casein (the commercial preparation, activin, was used), histamine, adrenalin, insulin, 0.3 per cent hydrochloric acid, 0.5 per cent hydrochloric acid, 1 per cent hydrochloric acid, 10 per cent hydrochloric acid.

It will be seen that substances representing fats, carbohydrates, proteins, metals and other chemicals were used in tests of their local effects. It was thought that perhaps chemicals of certain structure might act more specifically than others in producing gastric defects. As control, the effect of trauma alone was noted.

Determinations of the gastric acidities were made following histamine stimulation. It is important to call attention to the fact that the average acidities in most of our dogs following this stimulation varied but little. Even after the injection of substances into the stomach wall, no changes in acidity were noted. The resistance of the gastric tissues to the injected material varied considerably in different dogs. Where the dog is well nourished, its resistance is likely to be far better than in the underfed animal. Lowered resistance is recognized to be conducive to ulcer formation.

The dogs were operated upon under ether anesthesia. The stomach was exposed through a right rectus incision, care being taken to prevent unnecessary trauma. Injections of 0.5 cc. of various substances were made into the muscular coat of the lesser curvature using a hypodermic needle of very small gauge. In a few instances the greater curvature was selected for injection. The injections were made through the serosa, the stomachs not being opened. We made certain that the injected materials were placed in the muscular coat not only by the resistance offered to the needle but also by the bulge which appeared after each injection and in the case of lipiodol and bismuth by x-ray verification. The area injected was marked exactly by the placement of a colored bead. The beads were attached only to the serosa and care was always taken not to include any of the deeper structures. The lesser curvature was the site most frequently selected in these experiments since it is the favorite area of ulcer formation. In some instances, however, the greater curvature was selected and it is of interest to note (1) the difference in the resistance of the muscle layers to the hypodermic needle, and also (2) the difference in rapidity of diffusion of the injected substances in the two areas. In other words, it was found that the greater curvature offered less resistance and allowed the material to diffuse much more rapidly.

Utilizing the method of multiple injections for the detection of hypersensitivity to certain substances, just as in the case of the skin, we injected as many as four different materials into the stomach wall of the dog, placing them so that they did not interfere with one another. For example, two injections were made on the anterior wall and two on the posterior wall, each separated from the other by a considerable area. Colored beads were again used, each substance being represented by a distinct color. This made identification relatively simple.

The dogs were fed on the ordinary laboratory diet and there was no interference with their usual habits. When acid was administered, this was done through a

stomach tube. The animals were eventually killed with ether and autopsies were performed as routine. The stomach, after complete removal, was opened along the greater curvature and spread out. The mucosa was examined with meticulous care and the injected area corresponding to the bead, which was easily identified, was located and excised with a wide margin. Histological studies were then made.

EXPERIMENTAL

Under histamine stimulation numerous gastric analyses were performed in a large series of dogs and an average normal acidity established. Repeated examinations showed but slight variations from this average except in some instances in which excessive mucus was present. Instances in which this was the case were not included in obtaining the average normal.

After the injection of various substances into the stomach wall, gastric analyses were made at frequent intervals. We were unable to demonstrate any tendency toward hyperacidity, the average normal being about the same. Even after the formation of an acute ulcer, no increase in the acidity was observed.

Trauma.—As controls, beads were placed over areas traumatized by a threaded needle which was passed through the serosa. Upon subsequent examination the stomachs were found to be normal in every respect.

1. *Sterile Water.*—For this experiment four dogs were utilized. 0.5 cc. of sterile water was injected into the muscular coat of the lesser curvature of the stomach. After 17 days, two of the dogs were killed and on macroscopic examination the mucosa was found to be normal. This observation was verified by histological studies. The other two dogs were killed after 22 days, after having been fed 1100 cc. (60 to 120 cc. *q. d.*) of 0.3 per cent hydrochloric acid. This additional administration of acid did not produce any change in the end-result, since both microscopic and macroscopic examinations were negative.

2. *Normal Salt Solution.*—The experiments performed with sterile water were repeated, using normal (physiological) saline. The results obtained were also found to be normal.

3. *Glucose (50 Per Cent).*—With the use of this agent the findings were exactly similar to those obtained after the injection of sterile water and saline solution.

4. *Skimmed Milk*.—Five dogs were used in this experiment and after the 5th day three dogs were killed. These revealed slight submucosal edema and congestion, the mucosa remaining normal. The other two dogs having been fed with 800 cc. of 0.3 per cent hydrochloric acid were killed on the 15th day and no changes were detected.

5. *Whole Milk*.—Five dogs were utilized. Of three, which were killed after 5 days, there was found in two, a slight submucosal edema and congestion, while the remaining one revealed no abnormalities. The other two dogs had been fed with 15 cc. of 0.3 per cent hydrochloric acid twice daily for 15 days when they were killed. These showed no abnormal changes.

6. *Omnadin (a Combination of Proteins and Lipoids Used in Non-Specific Immunization Therapy)*.—Five dogs were used. In three, killed on the 5th day, the mucosa was normal; the submucosa showed in two instances a localized leucocytic infiltration with large phagocytic cells, while, in the other, no changes of this type were observed. The remaining two dogs were fed with 300 cc. of 0.3 per cent hydrochloric acid twice daily for 15 days and in these no changes were found.

7. *Lipiodol*.—Four dogs were utilized. In order to determine and verify the exact location of the injected material, the dogs were radiographed. It was found that the opaque substance was definitely located in the gastric wall. (Lipiodol was used to represent a foreign body, not easily absorbed and therefore possibly causing a prolonged irritative action by pressure.) Two dogs were killed on the 17th day. Macroscopically these showed large indurated nodules, 1 cm. in diameter, which represented the unabsorbed lipiodol. Further studies under the microscope gave no indication of tissue injury but showed large globules of unabsorbed lipiodol. The remaining two dogs had been fed daily with 30 cc. of 0.3 per cent hydrochloric acid for 21 days and despite the administration of this dilute acid the end-results were the same.

8. *Bismuth Subcarbonate*.—As another type of non-absorbable foreign body, bismuth was employed in a manner exactly identical with the lipiodol experiment. The final results were a repetition of those obtained with the lipiodol except that the bismuth microscopically revealed itself as a brownish black pigment.

9. *Pituitrin (S.)*.—Five dogs were utilized. One dog died after 4

days and revealed no gastric changes at autopsy. After 21 days, two dogs were killed and whereas macroscopic examination showed a slight redness and edema, the microscopic examination revealed a normal mucosa with only slight submucosal edema. After 25 days one dog was killed and similar findings were noted. The remaining dog was fed with hydrochloric acid, receiving 750 cc. of 0.3 per cent hydrochloric acid in small doses of 15 cc., and being killed after 15 days. Macroscopically, no abnormalities were observed but microscopically slight submucosal edema was found.

10. *Activin (5 Per Cent Cascin)*.—Six dogs were used. One died after 4 days and revealed no gastric changes at autopsy. In two dogs killed after 21 days, the mucosa was found to be normal except for slight redness over the injected area; the submucosa showed slight edema; otherwise, no abnormalities were noted. In one dog killed after the 25th day, a slight mucosal erosion was noted on both macroscopic and microscopic examinations of the stomach. In addition, vascular engorgement and submucosal edema were observed. Two dogs were fed with acid; one received 765 cc. of 0.3 per cent hydrochloric acid in small doses, and when killed on the 15th day, presented no gastric changes. The other, killed on the 14th day after having been fed with 350 cc. of diluted hydrochloric acid, likewise showed no pathological changes.

11. *Histamine*.—Six dogs were used. One died after the 4th day and revealed no gastric changes at autopsy. Two dogs, killed after 21 days, presented a normal gastric mucosa and a pronounced submucosal edema. In another dog, killed after the 25th day, there was found a small healed ulcer which on microscopical study revealed a slight mucosal erosion with infiltration of polymorphonuclear leucocytes and mononuclear cells in the stroma between the glands, engorgement of the blood vessels and moderate submucosal edema. In two other dogs, fed respectively with 350 and 765 cc. of 0.3 per cent hydrochloric acid, there were no macroscopic nor microscopic changes after 14 days.

12. *Adrenalin*.—Three dogs were used. In one dog, killed after 25 days, there was found on macroscopic examination a small congested area and on microscopic examination an infiltration between the glands, moderate engorgement of blood vessels and submucosal edema.

In the other two dogs, killed after 21 days, the mucosa was found to be normal and the submucosa slightly edematous. In each instance macroscopic swelling of the tissue corresponding to the injected area was observed.

13. *Insulin*.—Three dogs were used. One dog died after 4 days, and showed no gastric changes at autopsy. Two dogs were fed with 0.3 per cent hydrochloric acid, 350 and 765 cc. respectively, and revealed no microscopic nor macroscopic changes.

14. *0.3 Per Cent Hydrochloric Acid*.—Nine dogs were utilized in this experiment. In two dogs 0.5 cc. of 0.3 per cent hydrochloric acid was injected into the muscular layer of the lesser curvature of the stomach. These were killed after 16 days and on macroscopic and microscopic examinations no abnormalities were noted. After the injection of 0.5 cc. of 0.3 per cent hydrochloric acid into the muscular layer of the stomachs of three other dogs, they were, in addition, fed with 30 cc. of 0.3 per cent hydrochloric acid twice daily for 15 days. In two of these, definite small mucosal ulcers were noted and in the other no macroscopic nor microscopic changes were observed. As a check on these findings two other dogs were injected and fed with 0.3 per cent hydrochloric acid. After 12 days of this treatment the dogs were killed. One showed a definite small ulcer filled with necrotic tissue and leucocytes. In the other, the mucosa was found to be normal and the submucosa showed a moderate edema. In two dogs, 0.5 cc. of 0.3 per cent hydrochloric acid was injected into the muscular coat of the anterior aspect of the *greater curvature* near the pyloric antrum. On the 16th day these dogs were sacrificed and examination disclosed no abnormal changes. These dogs were not fed with acid.

15. *0.5 Per Cent Hydrochloric Acid*.—Two dogs were utilized. These dogs were not fed with acid. On the 9th day they were killed. In one, a definite deep ulcer was noted surrounded by an indurated edge. Microscopically the ulcer was surrounded by leucocytes and fibrin. The base was found to be infiltrated down to the muscular coat. The other showed a definitely thickened area but no ulceration nor erosion.

16. *1 Per Cent Hydrochloric Acid*.—Eight dogs were utilized in this experiment. Two were killed after the 9th day. No acid was fed to these dogs. In one of them, the macroscopic examination revealed a perforated ulcer walled off by omentum. Microscopically, the per-

forated ulcer was found to be surrounded with inflammatory exudate. In the other there was found a definite ulcer of the penetrating type verified by both macroscopic and microscopic examinations. In two dogs the injection of 0.5 cc. of 1 per cent hydrochloric acid was made into the muscular coat, on the anterior aspect of the greater curvature near the pyloric antrum of the stomach. On the 12th day these dogs were killed and no abnormal changes were observed. In the four remaining dogs, the injections of 1 per cent hydrochloric acid were again made into the muscular coat of the *greater curvature*. They were killed on the 17th day. One of these revealed a perfectly normal mucosa, while in three, definite penetrating ulcers were observed. In this series of experiments the animals had not been fed with acid.

When higher concentrations of HCl are employed (e.g. 10 per cent), either by introduction into the viscus or injection into the muscular coat, destructive effects are displayed which lead to perforating ulcers. The lesions are of acute character and should not be confused with the chronic ulcerating processes in human cases.

The Effect of Histamine Given Subcutaneously.—Acute ulcers were produced in four dogs by the direct injection of 1 per cent hydrochloric acid into the muscular coat of the stomach. It was thought that histamine given subcutaneously might cause the acute ulcer to become chronic by maintaining a maximum secretion of acid or by the toxic effect of the excessive amounts of histamine administered. To that end the four dogs were given 0.5 mg. of histamine subcutaneously, one to three times daily. One dog died 23 days following the production of the ulcer after receiving 54 injections of histamine. Autopsy disclosed a large penetrating punched out ulcer of the acute type. In the three remaining dogs which were killed on the 95th day after receiving 146 injections, autopsy revealed no ulcer in two and in the other a very superficial erosion was noted. In a second control group of four dogs acute ulcers were produced in the same way but in these no histamine was administered and small healed ulcers were observed in each instance probably illustrating the fact that subcutaneous injection of histamine did not have a significant influence upon the production of chronic ulcers but on the contrary seemed to favor complete healing. Despite the small number of dogs the results obtained pointed clearly, it seemed to us, to the fact that histamine given as described had no

pronounced effect upon acute ulcers. It is interesting that even after the continuous use of histamine over a long period of time the acidities were not markedly affected and the relationship between ulcer, healing ulcer, scar and acidity was too variable to be of any significance.

We are greatly indebted to Doctor Hugh R. Spencer of the Department of Pathology for his helpful advice and for his histological studies of our specimens.

DISCUSSION AND SUMMARY

Although other investigators have attempted to demonstrate that histamine has the power of producing acute ulcers when injected locally into the stomach wall, our experiments show clearly that this is a very inconstant finding for, of six instances, we were only able to obtain a small healed mucosal ulcer in one. Even when dilute acid (0.3 per cent hydrochloric acid) was fed to these animals they did not develop ulcers or erosions. It has also been stated that histamine, given subcutaneously, may produce ulcers secondarily to oversecretion of acid, especially when at the same time histamine has been injected into the stomach wall. We have been unable to confirm this observation.

In our series of experiments on dogs which were observed over a varying period of time ranging from 4 to 95 days we utilized other substances in addition to histamine. After establishing controls, sterile water, normal saline, 50 per cent glucose, skimmed milk, whole milk, omnadin, lipiodol, bismuth subcarbonate, pituitrin (S.), activin, histamine, adrenalin and insulin were injected into the muscular coat of the stomach and it was found that such procedures rarely produced any definite changes in the stomach mucosa even when supplemented by the feeding of dilute hydrochloric acid (0.3 per cent hydrochloric acid). We were able to demonstrate conclusively that the trauma involved in our operative procedure could not in itself account for any of the pathological findings. We were also able to show that the pressure due to the injected materials did not produce erosions or ulcerations. The injections of acids produced varying results. Diluted acid (0.3 per cent) injected into the stomach wall gave almost uniformly negative results but when combined with the feeding of acid of equal

strength erosions were occasionally produced. Our results with 0.5 per cent hydrochloric acid were also inconstant. The 1 per cent hydrochloric acid produced, in many instances, definite ulcerations even when not accompanied by the feeding of acid.

CONCLUSIONS

1. The various substances utilized in these experiments when injected into the muscular coat of the stomach wall did not have any significant relationship to the production of ulcer.

2. Weak solutions of hydrochloric acid had no corrosive effect upon the gastric mucosa and rarely produced ulceration when injected into the muscular coat of the stomach. Stronger solutions produced definite ulcerations.

3. No relationship could be demonstrated between the injection of various substances into the stomach wall and the production of hyperacidity.

4. The prolonged use of histamine administered subcutaneously was not a factor in the production of chronic ulcers even after 1 per cent hydrochloric acid was injected into the muscular coat of the stomach.

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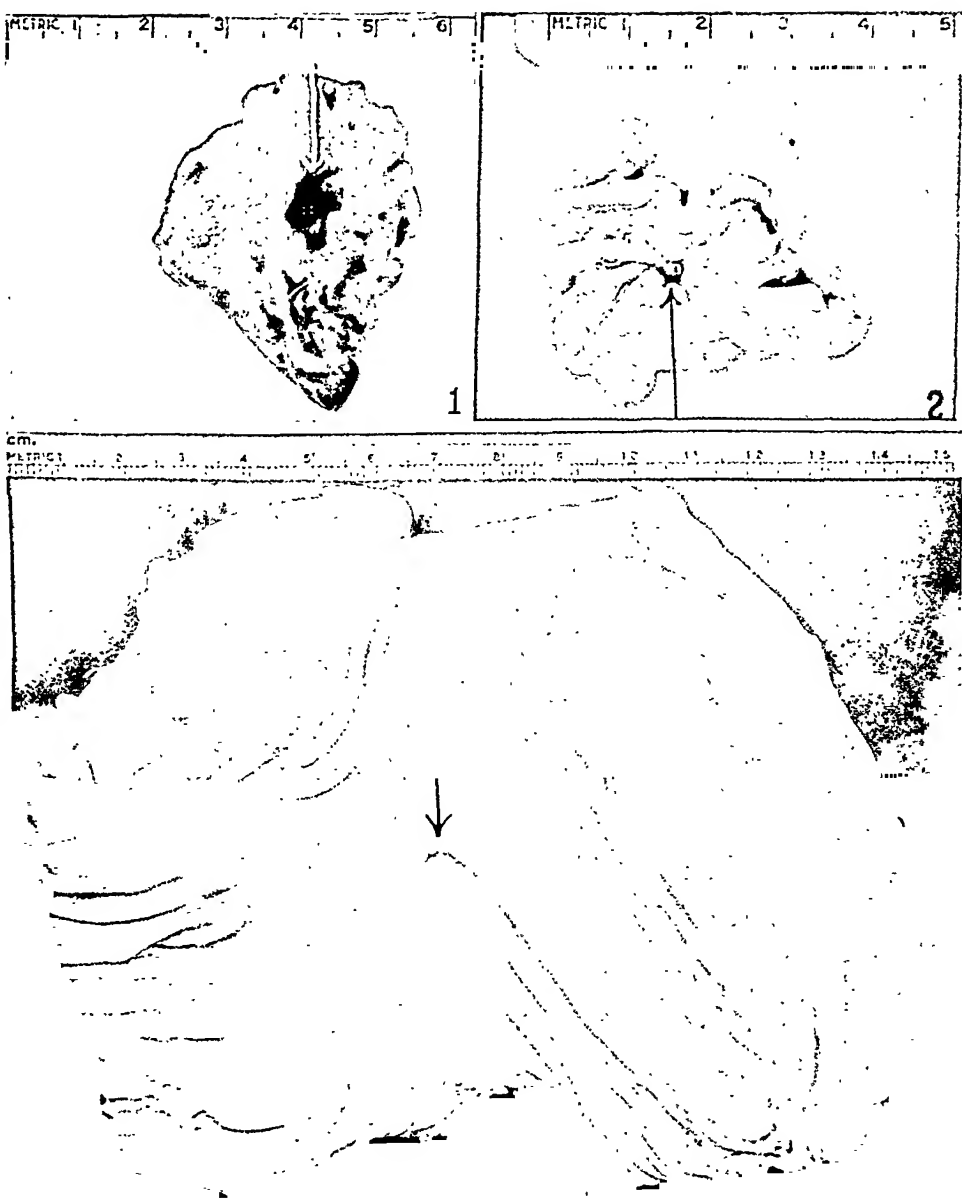
EXPLANATION OF PLATE 14

FIG. 1. A perforated ulcer, walled off by peritoneum, produced by the injection of 1 per cent hydrochloric acid. The dog was killed on the 8th day.

FIG. 2. A small penetrating ulcer, deep puckered type, with thickened elevated edge. Mucosal folds converge towards the ulcer. This ulcer was produced by

the injection of 1 per cent hydrochloric acid into the wall of the stomach. The dog was killed on the 19th day.

FIG. 3. Deep punched out ulcer of penetrating type, extending down into the muscularis produced after the injection of 1 per cent dilute hydrochloric acid into wall of stomach, following daily subcutaneous injections of histamine over a period of 23 days. This occurred in only one dog of the series. In another dog only a small mucosal erosion was encountered, while in two dogs a perfectly normal mucosa was found.



(Friedenwald *et al* : Acids in production of acute gastric ulcers)

LESIONS OF THE NERVOUS SYSTEM IN VITAMIN DEFICIENCY

I. RATS ON A DIET LOW IN VITAMIN A

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PLATES 15 TO 18

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Lesions of the peripheral nervous system have been recognized and described in animals fed diets deficient in the antineuritic vitamin B, but there has been relatively little written about nervous system lesions in association with diets deficient in vitamin A. Hart, Miller and McCollum (1) described histologic changes "not unlike those recorded for beri-beri" in the nervous system of swine fed certain wheat and grain mixtures, and they concluded that these changes were the result of toxic materials in the food rather than due to the absence of vitamin A. Steenbock, Nelson and Hart (2) described unsteadiness of gait in a dog fed a diet deficient in fat-soluble vitamin but made no mention of examination of the tissues of the nervous system. Mellanby (3-6) observed incoordination, spasticity and weakness in young puppies fed a diet of 10 per cent wheat germ which was at the same time deficient in fat-soluble vitamins. He described lesions consisting of scattered degeneration in the spinal cord and observed that the ascending fibers were particularly involved. He related these lesions to a positive harmful influence in the wheat germ in the absence of a defending chemical substance like vitamin A. More recently Suzman, Muller and Ungley (7) have described experiments in which adult dogs were fed a diet abundant in cereal and lacking in vitamin A conducted with the idea of attempting to produce lesions in the spinal cord, but they did not succeed in accomplishing this end.

In the Laboratory of the Department of Obstetrics in this institution, Dr. S. B. D. Aberle has been conducting experiments with rats on a diet deficient in vitamin A with no direct intention of observing nervous system lesions or manifestations. It was observed, however, in the course of the feeding that certain animals developed marked weakness and incoordination of the extremities. As a result of this observation we undertook a study of the tissues of the central and peripheral nervous systems of these animals. It is the purpose of the

present communication to present the results of the studies made on the tissues of the central and peripheral nervous systems of many of these rats.

Experimental Procedure

Diet Employed.—All the animals used in this study, with the exception of certain controls, were placed on an artificial ration deficient in vitamin A when 21 to 24 days of age. The composition of the ration was:

	<i>per cent</i>
Casein.....	15-18*
Corn-starch.....	56-63*
Crisco.....	18-22*
Osborn-Mendel Salt Mixture IV.....	4

* It was found that the animals ate better when the proportion of the various constituents of the diet was changed weekly or biweekly. This was done within the limits indicated in the table.

The vitamin B complex was supplied in the form of yeast in quantity calculated and found by practice to be sufficient for the needs of this animal. Vitamin D was supplied in the form of irradiated ergosterol or irradiated yeast. Vitamin E was present in sufficient quantity in the crisco; vitamin C is apparently not needed by the rat.

Material Studied and Technic Employed.—The nervous systems of a total of twenty-three animals were examined. The animals were grouped into seven classes in accordance with conditions of experiment to be specified for each group.

Blocks of the brains and spinal cords were fixed immediately upon removal from the animals in 95 per cent alcohol, in a solution of formaldehyde, U.S.P. (1:10), and in Müller's solution. The brachial plexuses, the sciatic and vagus nerves of each animal were fixed in Müller's and in formaldehyde solution, as were the optic nerves from five of the rats in Group A. The alcohol-fixed material was embedded in celloidin and stained by the original Nissl method (toluidine blue). The formaldehyde-fixed material was stained for fat by the Scharlach R method and for the demonstration of myelin sheaths by the Spielmeyer method. The material fixed in Müller's solution was stained with osmic acid, embedded rapidly in celloidin and sectioned at 30 microns. In many instances the spinal cords were sectioned longitudinally as well as transversely, and an average of four blocks at different levels were taken from each cord. About ten sections in serial were cut from each block and mounted for histologic examination.

RESULTS

On *gross* examination no abnormalities were found in the brain, spinal cord or peripheral nerves of any of the animals.

In none of the animals except Rat 76 (Group A) were there any lesions in the *brain* demonstrable microscopically by any of the staining methods. In that animal the ventral pontile nuclei contained many vacuolated nerve cells in the Nissl preparations, but the Spielmeyer, Marchi and Scharlach R preparations were negative.

None of the optic nerves examined showed any pathologic change grossly or microscopically.

Group A. Nine Rats on Diet Deficient in Vitamin A, from Mothers on Diet of 100 Per Cent Calf Meal during Pregnancy and Lactation.—

Rat. 65.—This animal was on the special diet for a total of 54 days and had paralysis for a period of 20 days, at the end of which time it died. Its maximum weight was 81 gm., but at necropsy it weighed only 65 gm.

Nissl preparations of the spinal cord showed swelling and cytolysis of the anterior motor horn cells, but there was no increase in the glia. In the Marchi preparations there was much black pigment deposition at the points of entrance of the posterior nerves into the cord. Somewhat less pigment was scattered in the posterior columns and was also present in an irregular manner around the periphery of the cord. Much degeneration was present in the posterior nerve roots and to a somewhat less degree also in the anterior roots. Scharlach R and Spielmeyer preparations of the spinal cord showed no degeneration. The vagus and sciatic nerves and those of the brachial plexus showed degeneration of an equal degree by all the staining methods (Fig. 2).

Rat 71.—This animal was on the special diet for 44 days and was paralyzed for 9 days, when it was killed. It had lost 14 gm. of weight from a maximum of 81 gm.

The anterior motor horns contained swollen, chromatolytic nerve cells. There was no proliferation of glia. Marchi preparations showed numerous black granules on the periphery of the ventral side of the cord and only a few granules in the posterior columns. Granules were absent in the nerve roots. Scharlach R and Spielmeyer preparations of the cord showed no myelin degeneration. The peripheral nerves were the seats of extensive demyelination (Figs. 1 and 3).

Rat. 72.—This rat was on the special diet for a period of 72 days and was paralyzed for 33 days, when it died. Its maximum weight was 102 gm. and at necropsy it weighed but 77 gm.

A few ganglion cells in the anterior motor horns of the spinal cord showed chromatolytic changes. Marchi preparations of the cord were not made. Spielmeyer stains revealed large clear vacuoles indicating degeneration on the margins of the cord. In one Scharlach R preparation there was found a collection of fat droplets in the white matter. Extensive demyelination was demonstrable in the brachial plexuses and sciatic nerves by both the Marchi and Scharlach R methods. The vagi showed no involvement.

Rat 73.—This rat was on the artificial diet for 53 days and was paralyzed for

14 days, when it was killed. It had maintained its weight fairly constantly at the maximum of 76 gm.

There were no changes in the spinal cord demonstrable by the Nissl technic, and Marchi preparations of the cord were not made. In the Spielmeyer stains there were present large clear vacuoles on the periphery; Scharlach R preparations failed to reveal fat. By the latter staining method, however, one of the dorsal nerve roots was shown to contain an abundance of brilliant red particles. All the peripheral nerves, including the vagi, were strongly positive for fat by both the Scharlach R and Marchi methods (Fig. 4).

Rat 74.—The total experimental period of this animal was 73 days, during the last 30 days of which it was paralyzed. This animal had lost but 7 gm. in weight from a maximum of 103 gm. It was killed.

The Nissl preparations of the spinal cord were negative. Marchi preparations of the cord were not made. By the Spielmeyer method there was once more observed a looseness of structure and vacuolization on the periphery. In this cord the vacuolization was also present symmetrically in the posterior columns. Scharlach R preparations were completely negative. Degeneration of marked degree was demonstrated by the Marchi method in the sciatic nerves and to a less degree in the nerves of the brachial plexus. The vagi showed no degeneration by this method. The distribution of the myelin degeneration could be confirmed in the Scharlach R preparations.

Rat 75.—This animal was on the special diet for 55 days and showed symptoms of paralysis for 6 days, at the end of which time it died. Its maximum weight was 120 gm. and at necropsy its weight was 108 gm.

Nissl preparations of the cord were negative; Marchi preparations were not made. By the Spielmeyer method there could be shown vacuoles on the periphery, but no fat could be demonstrated by the Scharlach R stain. Fatty degeneration of the myelin sheaths of the brachial plexuses and sciatic nerves were found in the Marchi and Scharlach R preparations.

Rat 76.—This rat was on the experimental diet for 71 days and was paralyzed for a period of 22 days, at the end of which it died. Its loss of weight amounted to 10 gm. from a maximum of 107 gm.

A few anterior motor horn cells in the spinal cord showed chromatolytic changes and one glia rosette was present in the crossed pyramidal tract. In the Spielmeyer preparations the myelin sheaths on the periphery of the cord appeared spongy and vacuolated, but Scharlach R preparations did not disclose the presence of fat. In both Marchi and Scharlach R preparations there was present some fatty change in the brachial plexuses and sciatic nerves. The vagi were not examined. The Spielmeyer preparations confirmed the presence of degeneration observed by the other methods.

Rat 78.—The total duration of the experiment was 53 days and the animal was paralyzed the last 20 days of its life. This animal was killed, having lost no weight.

There were no changes observed in the nerve cells of the spinal cord. There were demonstrated large vacuoles on the periphery of the cord by the Spielmeyer technic (Fig. 9), but Scharlach R preparations of the cord and the nerve roots were negative for fat. All the peripheral nerves, the vagi, brachial plexuses and sciatics, were found to contain moderate numbers of black granules in the Marchi stains.

Rat 79.—This rat was on the diet for 50 days and had paralysis for 18 days, at the end of which time it was killed. The animal gained weight steadily up to the time of its death, when it weighed 85 gm.

There were no changes in the spinal cord that could be demonstrated by the Nissl stains. In the Spielmeyer preparations of the cord there was seen marked degeneration of the posterior columns and to a less extent also on the periphery (Fig. 10). Scharlach R preparations failed to show fat; Marchi preparations were not made. Extensive degeneration of the medullary sheaths was present in the brachial plexuses and the sciatic nerves, but the vagi were completely normal.

Comment

In each of the nine rats lesions were found in the spinal cord consisting of degeneration of the medullary sheaths of the sensory tracts on the periphery. In four of the animals the posterior columns showed degeneration and in two, the entering posterior nerve roots. These changes were demonstrated by the Spielmeyer myelin sheath stain and by the Marchi method. In Rat 72, which was paralyzed for 33 days, fatty change was demonstrated in the white matter of the cord by the Scharlach R stain. In Rat 65 there was degeneration of the anterior nerve roots in addition to the posterior. It should be mentioned that the findings in the nerve roots were discovered quite by accident since no systematic attempt was made to include these in the blocks taken for sectioning. The lesions that were observed in them suggest the interesting possibility that the changes in the cord were secondary to and dependent on them.

Every one of the animals showed extensive degeneration¹ of the medullary sheaths of the brachial plexuses and sciatic nerves; the vagi were degenerated in only four of the animals, and in another they were not examined. From the fact that the degeneration in the peripheral nerves could be demonstrated by the Scharlach R method in addition to the Marchi and Spielmeyer methods, the conclusion must be drawn that it was of longer duration in them than in the spinal cord. This

¹ This process is often designated by the term polyneuritis in the literature.

would support the view that the peripheral nerves were primarily involved, and that the demyelination continued to the spinal nerve roots from whence it spread to the spinal cord itself.

Four of the nine animals of this group showed changes in the ganglion cells of the spinal cord that were described as swelling, cytolysis or chromatolysis. It becomes at once apparent from a glance at the histories of these animals that the neuronal lesions were in no way dependent on the duration of paralysis or on the loss of weight. The fact was brought to light, however, that these changes occurred only in those animals which were necropsied after a lapse of several hours and not in those necropsied immediately after death. The same observation was made subsequently in some of the other animals of this study. In passing, it must be mentioned that similar changes in nerve cells are produced when toluidine blue (or even thionin and cresyl violet) is used to stain tissue fixed in a solution of formaldehyde instead of 95 per cent alcohol.

Group B. Two Rats on Diet Deficient in Vitamin A, from Mothers on Diet of Table Scrap during Pregnancy and Lactation.—

Rat 122.—This animal was on the special diet for a total of 98 days and showed paralysis only on its last day of life. Its maximum weight was 122 gm. and at necropsy it weighed 96 gm. It was killed.

Nissl preparations of the spinal cord were completely negative. Marchi preparations of longitudinal sections of the cord showed an exquisite picture of degeneration of the posterior columns, the motor tracts and the posterior nerve roots as they entered the cord (Fig. 6). The anterior roots were likewise involved, but to a much less degree. Scharlach R preparations of the spinal cord were entirely free of fat. Large vacuoles were present in the posterior columns and at the periphery of the cord in the Spielmeyer stain. A striking picture of degeneration of the myelin was seen in the peripheral nerves by the Marchi method. The Spielmeyer and Scharlach R preparations of these nerves, however, were negative.

Rat 125.—This animal was on the diet deficient in vitamin A for a period of 105 days. It displayed signs of paralysis for a period of 20 days, at the end of which time it was killed. Its maximum weight was 196 gm. and at necropsy it weighed 154 gm.

The brain and spinal cord of this animal were not studied. Severe degeneration of the medullary sheaths of the brachial plexuses and sciatic nerves was found in the Marchi stains, but the other staining methods yielded negative results.

Comment

It will be noticed that the two animals of this group, from mothers on a diet of table scrap which is adequate in vitamin A, developed signs

of paralysis at a much later time than the animals in Group A of this study. This merely confirmed the well known fact that animals are born with vitamin A stored in their tissues if their mothers receive an adequate supply of this vitamin during pregnancy. The lesions in the peripheral nerves of these two animals were similar to those observed in the rats of Group A, but perhaps they were earlier lesions since they could be demonstrated in Marchi preparations only. It is of great interest to note that in the spinal cord of Rat 122 there was degeneration present in the motor as well as in the sensory tracts. It would seem that the lesions in the cord followed those in the peripheral nerves on the sensory side, and produced those in the peripheral nerves on the motor side.

Controls

Group C. Two Rats on Diet Deficient in Vitamin A, from Mothers on Diet of Table Scrap during Pregnancy and Lactation. Animals Killed before Any Signs of Vitamin A Deficiency Appeared.—Rats 132 and 133.—Each animal was killed 35 days after being placed on the special regimen. The first animal gained 67 and the second gained 76 gm. during this period. There were no changes of any kind in the spinal cords which could be demonstrated by any of the staining methods. Similarly, the brachial plexuses, and the vagus and sciatic nerves were negative for any lesions in their medullary sheaths.

Comment

In spite of prolonged subsistence on a diet deficient in vitamin A, the two animals of this group failed to show any changes in their nervous systems at necropsy. They were killed before showing any signs of the deficiency disease, which would indicate a close parallel between the clinical manifestations of nervous lesions and the anatomic changes responsible for them.

Group D. Five Rats on Diet Deficient in Vitamin A. Animals Partially or Completely Cured of Symptoms of Paralysis.—Rat 68.—This animal developed signs of paralysis after 33 days on the special diet. These lasted for 34 days, to disappear with the repeated administration of cod liver oil. The animal was killed 3 days after the disappearance of the signs of paralysis. Lesions were not found in the spinal cord by the Nissl technic. Tremendous vacuolization was present in the posterior columns and at the periphery of the cord in both Spielmeyer and Scharlach R preparations. Marchi stains were not

made of the cord. Definite but not very extensive degeneration of the myelin sheaths of the peripheral nerves could be demonstrated by the Marchi method. This finding, particularly in the sciatic nerves, was confirmed in the Spielmeyer and Scharlach R preparations.

Rat 77.—Signs of paralysis appeared after 43 days on the special diet, and persisted up to the time the animal was killed, which was 29 days later. However, there was progressive improvement in the signs with the repeated administration of cod liver oil.

Changes in the spinal cord could not be demonstrated by the Nissl method. Vacuolization of the posterior columns and periphery of the cord was pronounced in the Spielmeyer preparations. Although the vacuoles were demonstrable in the Scharlach R preparations, no fat was found. Marchi preparations of the cord were not made. A moderate amount of degeneration was found in the peripheral nerves by the Marchi method, but the other stains were negative.

Rat 115.—This animal failed to develop any signs of paralysis (its mother was on a table scrap diet) after being on the special diet for 58 days. It was given cod liver oil, nevertheless, for a period of 12 days, at the end of which it was killed.

The spinal cord was not examined. The peripheral nerves showed distinct but not very extensive myelin degeneration in the Marchi preparations and to a less extent also in the Scharlach R and Spielmeyer preparations.

Rat 205.—This animal developed paralytic signs after 48 days on the diet, and these signs lasted for 30 days, to disappear after the administration of cod liver oil over a period of 20 days. The rat was killed 4 days after the disappearance of the paralytic signs.

The spinal cord appeared to be normal in the Nissl stains. In the Marchi preparations there was evidence of degeneration in the crossed and uncrossed pyramidal tracts, and to a slight extent also in the posterior columns and at the periphery of the cord (Fig. 8). Degeneration of one of the posterior nerve roots could be traced into the cord. In the Spielmeyer stains there was found vacuolization in the zones corresponding to the black granules seen in the Marchi preparations. The Scharlach R preparations were entirely negative. Moderately extensive degeneration was found in all the peripheral nerves by the Marchi method.

Rat 206.—This rat developed paralytic signs after 52 days on the diet, and these disappeared at the end of 26 days following the administration of cod liver oil for a period of 22 days. The rat was killed 4 days after the disappearance of paralytic signs.

Nissl preparations of the spinal cord revealed an entirely normal picture. In the Marchi preparations of cross-sections of the spinal cord there was found extensive degeneration of the periphery and of one of the posterior columns (Fig. 7). At another level the crossed and uncrossed pyramidal tracts showed degeneration in addition to the sensory tracts. The degeneration could be confirmed in longitudinal sections of the cord. There was present some vacuolization in the Spielmeyer preparations in those regions in which myelin degeneration was demon-

strable by the Marchi method. Scharlach R stains, however, were entirely negative for fat. The peripheral nerves showed well marked degeneration in the Marchi preparations (Fig. 5).

Comment

Rats 68, 77, 205 and 206 showed signs of paralysis for periods varying from 26 to 34 days. Three of the rats recovered from the paralysis with the repeated administration of cod liver oil, and they were killed 3 or 4 days later in each instance. Rat 77, although showing definite improvement, failed to recover completely from the paralysis. In view of the improvement noted in the symptomatology it is surprising that at necropsy such extensive lesions were present in the spinal cords and peripheral nerves of these animals. Similar observations have been made in experiments on vitamin B deficiency where it was found that the administration of yeast concentrate to paralyzed animals produced an almost immediate recovery. Obviously the clinical improvement of these animals could not be caused by a sudden restitution of the injured nervous systems to normal. Indeed, on the basis of such observations some workers have concluded that the paralysis was not caused by the peripheral polyneuritis. Such a view, however, seems to be unfounded.

The conclusion must be drawn from the present experience that it is possible to have little or no clinical evidence of nervous disease in the presence of even marked anatomic lesions in the nervous system during a certain period of recovery. What the anatomic picture in the nervous system would have been 14 or 30 days after recovery is obviously not answered by this "acute" experiment.

Again in Rat 205 there was evidence to suggest that some of the changes in the spinal cord were merely an extension of those in the nerve roots. In this animal and in Rat 206 the crossed and uncrossed pyramidal as well as the sensory tracts showed some degeneration. The problem presented by Rat 115 is unique in this study. The animal had been on the diet deficient in vitamin A for 58 days without presenting clinical evidence of injury to the nervous system. It did, however, show evidence of dietary deficiency by the presence of cornified epithelium in the vaginal smears. At necropsy the peripheral nerves showed distinct but not extensive demyelination. It may

possibly be supposed that the animal would shortly have developed paralysis had it not been killed or given cod liver oil.

Group E. Two Rats Fasted but Receiving Full Ration of Vitamins Including Cod Liver Oil.—

Rat S74.—This animal received but 1 gm. daily of the diet deficient in vitamin A in addition to the full ration of vitamins including cod liver oil. It died at the end of 17 days without developing signs of paralysis and after losing 53 gm. in weight.

There was a slight paleness at the periphery of the spinal cord but no vacuolization in the Spielmeyer preparations. The Scharlach R and Marchi preparations were entirely negative for myelin degeneration. The peripheral nerves presented an entirely normal picture by all the staining methods employed.

Rat S76.—This rat also received 1 gm. daily of the special diet in addition to the full ration of vitamins including cod liver oil. It died at the end of 16 days on this diet without showing signs of paralysis and after having lost 58 gm. in weight.

All the preparations of the spinal cord presented an entirely normal picture, the cord having been sectioned longitudinally and transversely. The peripheral nerves were free of degeneration except for one small zone in the Marchi preparations, where a few black granules were found.

Comment

The anatomic findings in these two animals showed fairly conclusively that inanition itself was not a factor in the production of lesions in the nervous system. Moreover, the two rats in this experiment lost much more weight than any of the rats in the other experiments. It was shown by Woollard (8) and by Zimmerman and Burack (9) that demyelination of the peripheral nerves was present in animals that were starved except for vitamin B, which was given in adequate amounts. It is noteworthy that these animals were at the same time deprived of vitamin A, which factor may have played an important rôle in the production of the lesions.

Group F. One Rat on Diet Deficient in Vitamin A Supplemented with Cod Liver Oil. Mother on Diet of 100 Per Cent Calf Meal during Pregnancy and Lactation.—

Rat 64.—This animal was on the special regimen for 111 days, during which time it increased its weight from 41 to 162 gm. At no time did it show paralytic signs.

A complete investigation of the nervous system, central and peripheral, by the various histologic methods employed in this study revealed a normal picture throughout.

Comment

That the artificial diet employed in this study when supplemented with an adequate amount of vitamin A was capable of maintaining rats in a good state of nutrition and did not cause nervous lesions even when fed over a long period of time, was shown by this experiment.

Group G. Two Rats on Diet of Table Scrap, from Mothers on Same Diet.—

Rats 164 and 165.—The first animal was killed when 89 and the second when 90 days old.

A complete investigation of the entire nervous system by all the histologic methods employed in this study revealed no pathologic changes.

DISCUSSION

The pathogenesis of lesions associated with vitamin deficiencies is not understood at the present time. According to some of the investigators mentioned previously, the lesions observed in animals fed diets rich in cereals and deficient in vitamin A are the result of injurious agents in the form of assumed "toxic substances" which act in the absence of a defending chemical substance like vitamin A. Since the diet employed in this investigation did not contain cereals, it is impossible to attribute the lesions to substances contained in these foods. In this connection it is of interest to consider the experimental results of Burr and Burr (10, 11) who employed rats to observe the effect of feeding a diet poor in unsaturated fatty acids. Although they do not mention physiologic or anatomic evidence of lesions in the nervous system, they describe urinary symptoms and changes in the tail and skin which they regard as significant since the diseased conditions cleared up when unsaturated fatty acids were added to the diet. These investigators found that it was impossible to produce the changes when corn-starch was one of the components of the diet. Since the ration fed the rats employed in the investigation presented in this communication contained corn-starch and, moreover, since none of the rats developed symptoms like those described by Burr and Burr, it seems reasonable that one might eliminate the possibility of such a deficiency. Thus it seems that the diet employed in these experiments is adequate in all respects excepting its content of vitamin A. However, since we have no information which suggests a possible mechanism for the development of lesions in vitamin deficiencies, the question as to whether

their presence merely serves as a protective one cannot be answered at this time. Experiments are now in progress in this laboratory which we hope may yield results that will throw some light on this phase of the subject.

With regard to the lesions described, it seems desirable to emphasize the importance of several staining methods. In the experience of the present writer, lesions of a degenerative nature in the nervous systems of experimental animals have so frequently presented difficulties of interpretation that it seemed fully justifiable to attempt to confirm them by the various staining methods employed. This was calculated to give both a positive (in the Marchi and Scharlach R preparations) and a negative picture (in the Spielmeyer preparations) of any possible degeneration in the medullary sheaths. Lesions of this kind which have been described in this communication have thus been confirmed by at least two methods of staining.

SUMMARY AND CONCLUSIONS

Under the conditions of these experiments, which consisted essentially of maintaining rats on a ration adequate in all dietary essentials as far as is known except vitamin A, the following changes were produced in the nervous system.

1. Degeneration of the medullary sheaths of the brachial plexuses and sciatic nerves, and less often of the vagus nerves. Such lesions were not found in the optic nerves.

2. Degeneration of the medullary sheaths of the sensory tracts on the periphery of the spinal cord and in the posterior columns. Much less frequently similar lesions were found in both the crossed and uncrossed pyramidal tracts.

3. Changes of the same nature in the posterior nerve roots and less frequently in the anterior nerve roots of the spinal cord. Evidence was adduced to indicate that the changes in the sensory tracts of the spinal cord followed those in the posterior nerve roots.

With the onset of muscular weakness and incoordination in these animals anatomic changes like those just described were found at necropsy, but they were not present for any appreciable period preceding the onset of these clinical signs.

For a short but undetermined period following clinical signs of

recovery from the nervous disease, marked lesions were still present in the nervous system at necropsy.

These lesions in the nervous system were produced by a ration containing no cereals which might have contributed a "toxic" substance to account for the degeneration of the myelin sheaths. Neither does a deficiency in unsaturated fatty acids appear to have played a rôle in their development.

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EXPLANATION OF PLATES

- PLATE 15
- FIG. 1. Rat 71. Drawing of sciatic nerve. Scharlach R stain. $\times 450$.
 FIG. 2. Rat 65. Drawing of sciatic nerve. Much of the fat is phagocytosed. Scharlach R stain. $\times 450$.

PLATE 16

- FIG. 3. Rat 71. Photomicrograph of sciatic nerve. Spielmeyer stain. $\times 455$.
 FIG. 4. Rat 73. Photomicrograph of sciatic nerve. Marchi stain. $\times 120$.
 FIG. 5. Rat 206. Photomicrograph of sciatic nerve. Marchi stain. $\times 120$.
 FIG. 6. Rat 122. Photomicrograph of longitudinal section of spinal cord showing degenerated myelin sheaths in posterior nerve root and in posterior column. Marchi stain. $\times 120$.

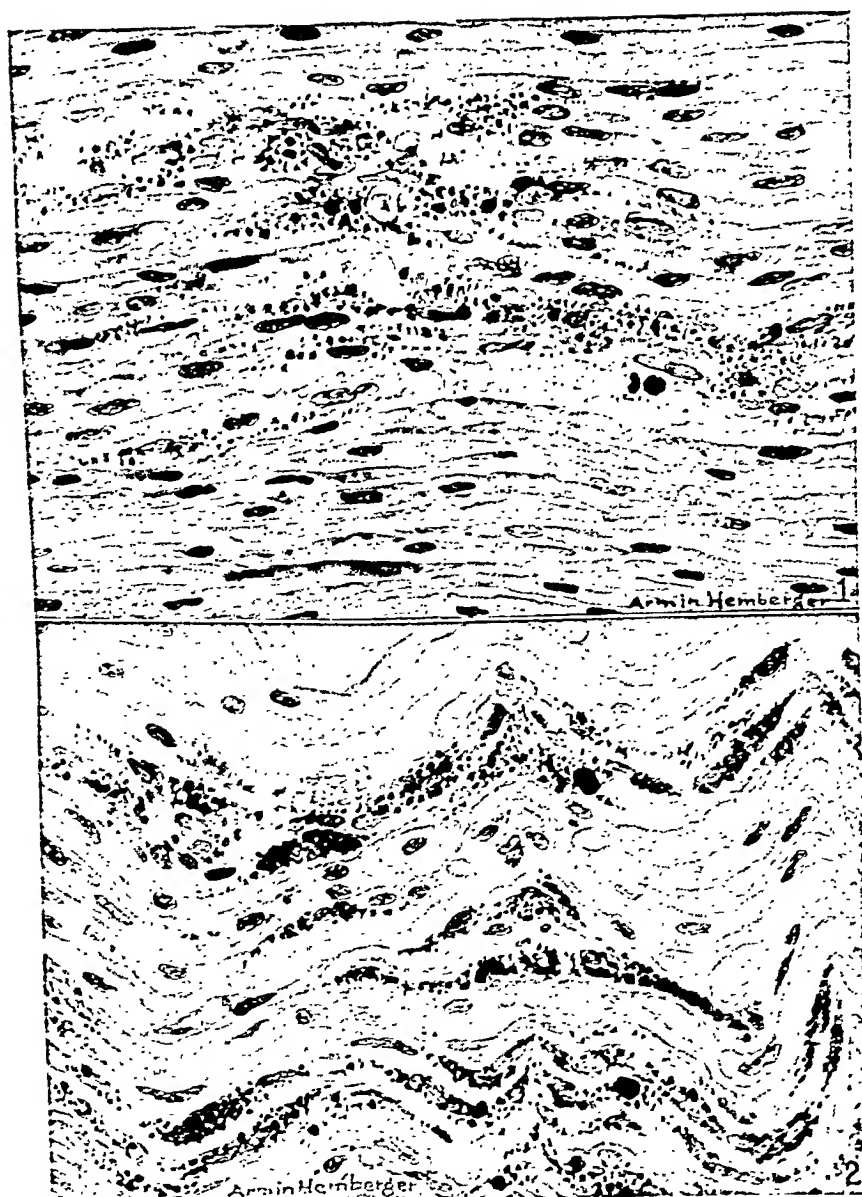
PLATE 17

- FIG. 7. Rat 206. Photomicrograph of transverse section of spinal cord. Note the marked degeneration in the posterior columns on one side and on the periphery of the cord. Marchi stain. $\times 30$.
 FIG. 8. Rat 205. Photomicrograph of transverse section of spinal cord showing degeneration of myelin sheaths in the anterior and lateral columns and on the periphery. Marchi stain. $\times 35$.

PLATE 18

FIG. 9. Rat 78. Photomicrograph of transverse section of spinal cord. The vacuolated regions on the periphery corresponded to those containing black granules in the Marchi preparations. Spielmeyer stain. $\times 40$.

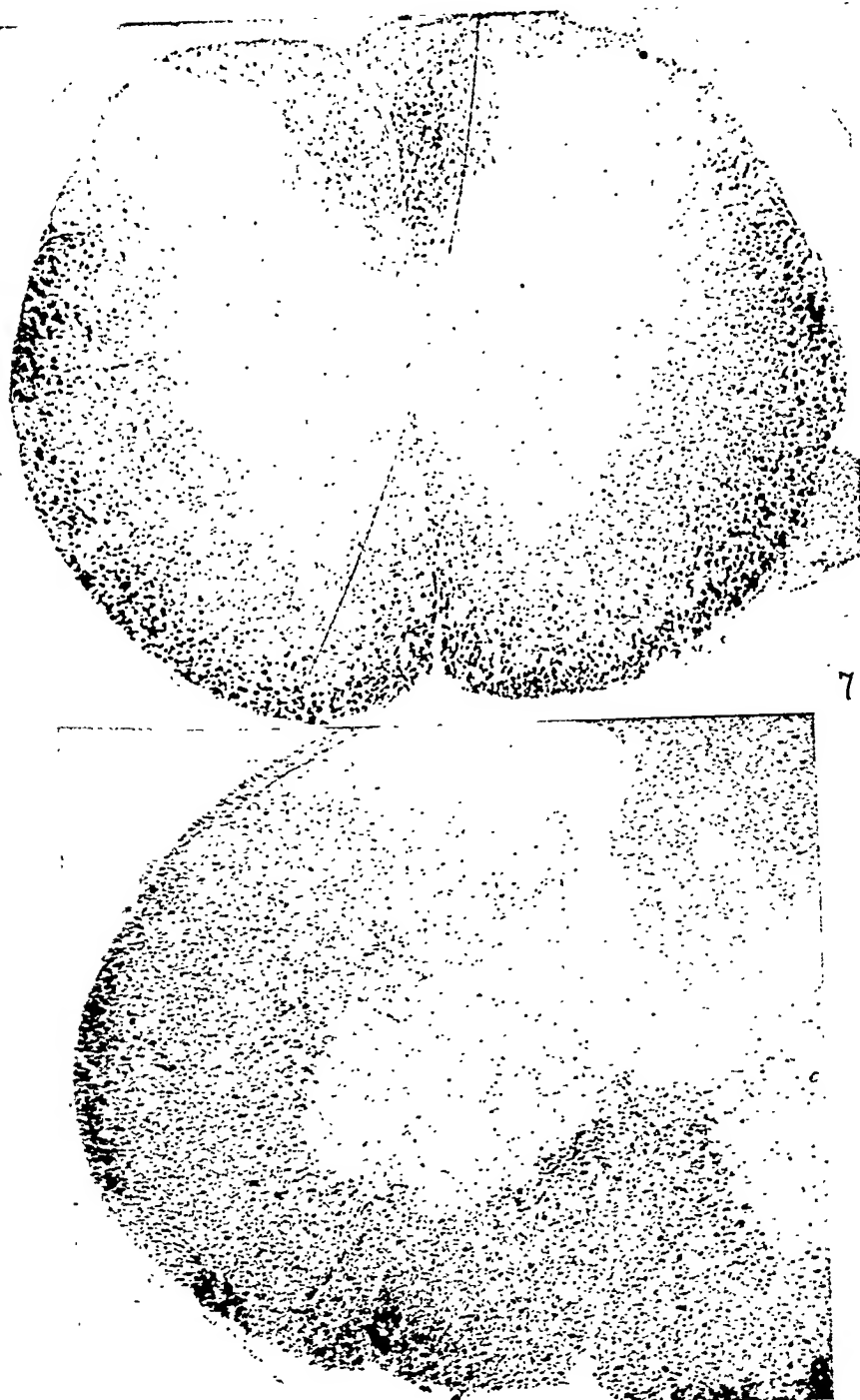
FIG. 10. Rat 79. Photomicrograph of transverse section of spinal cord showing marked degeneration of the medullary sheaths in the posterior columns and on the periphery. Spielmeyer stain. $\times 35$.



(Zimmerman: Nervous system in vitamin deficiency. 1)



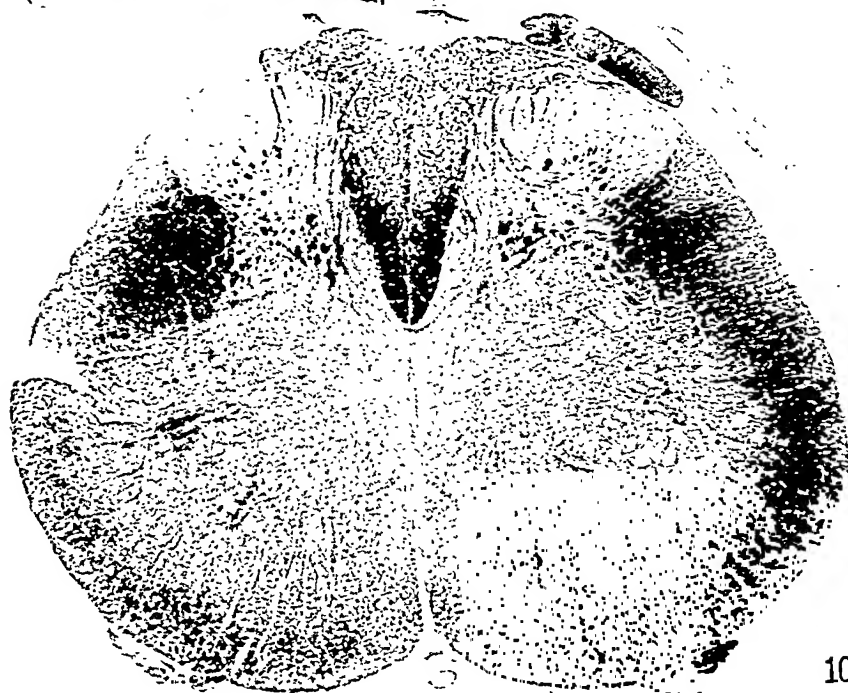




(Zimmerman: Nervous system in vitamin deficiency. 1)



9



10

STUDIES ON THE ETIOLOGY OF SPONTANEOUS CONJUNCTIVAL FOLLICULOSIS OF MONKEYS*

I. TRANSMISSION AND FILTRATION EXPERIMENTS

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The *Macacus rhesus* monkey is ordinarily employed for experimental transmission of human trachoma and for study of the inoculation effects of organisms derived from trachomatous tissues. There occurs, however, among certain stock monkeys, spontaneous conjunctival folliculosis which may simulate the experimental disease induced by the inoculation of suspensions either of human trachomatous tissues or of *Bacterium granulosis* (1, 2). Nevertheless, as has been mentioned previously (1, 3), it is possible to obtain reliable experimental results with trachomatous material by the selection of animals with clear conjunctivae, and their careful quarantine.

Spontaneous conjunctival folliculosis of monkeys was described by Hess and Römer in 1906 (4). They believed that it differed from experimental trachoma, as induced by inoculation of suspensions of human trachomatous tissues, in that it exhibited less numerous, smaller, and more superficial follicles, with less destruction of epithelium. They stated that while spontaneous folliculosis did not resemble human trachoma, either clinically or anatomically, experimental trachoma showed a close similarity to the disease in man.

The spontaneous conjunctivitis was also known to Nicolle and Lumbroso (3, 5) who reported in 1926 that by scarification of the palpebral conjunctiva of an affected eye, the lesions spread and after a short period of time follicles appeared in the conjunctiva of the other eye. The follicular contents, when inoculated into normal conjunctivae of fresh monkeys, induced a similar reaction; yet these investigators believed that there might be a "telluric" origin of the disease.

Wilson (6), in 1928-29, failed to confirm Nicolle and Lumbroso's finding of the spread of follicles by mechanical means and suggested, on the basis of clinical

* We owe many thanks to Dr. Ralph E. Knutti for his cooperation, especially in the earlier stages of these investigations.

experience, that the condition might not be telluric but infectious in origin. He studied fifteen monkeys of different species kept in the Zoological Gardens at Cairo, Egypt, of which twelve manifested folliculosis in either of two forms: Type I showed simple, non-inflammatory, discrete follicles scattered over the palpebral conjunctiva and probably enlarged lymphoid nodules, and Type II "inflammatory" conjunctivitis characterized by viscid secretion and larger follicles which were at times hemorrhagic. In neither case were the follicles present over the tarsal plate. Wilson regards as criteria for the diagnosis of trachoma, the presence of follicles over the tarsal plate; their rupture either spontaneously or on pressure; conjunctival scarring; and corneal pannus. In view of the fact that he found none of these signs in the spontaneous disease, he considered the latter not as trachoma but as folliculosis. Yet he could make no histological distinction between the two in their early stages.

Weiss (7) (1930) and Thygeson (8) (1932) also described spontaneous folliculosis, although Thygeson met with it in only three *Macacus rhesus* monkeys. The lesions were unaccompanied by signs of inflammation, their contents failed to infect normal monkeys, traumatism did not cause their generalization, and finally, the follicles were rapidly absorbed, leaving the conjunctiva normal. The histological structure of the spontaneous disease differed from that of experimental trachoma in being less pronounced in degree and giving rise to less cicatricial tissue and involvement of the tarsus.

Our own observations have shown that folliculosis, especially Type I of Wilson (6), is widespread among certain groups of animals received from dealers, particularly those housed under crowded and unclean conditions.

The foregoing review indicates that a divergence of opinion exists with respect not only to the cause of spontaneous folliculosis but also to its clinical and pathological identity. We undertook its study with the aim of determining, if possible, the incitant. In this first paper we shall describe the clinical and pathological appearance of the spontaneous disease and deal with the problem of whether it is disseminated by an infectious agent, and if so, under what conditions. The results here reported are based on observations upon over 250 *Macacus rhesus* monkeys.

Types of Spontaneous Conjunctival Folliculosis

Confirming the observations of Wilson (6), we have found that spontaneous folliculosis may exist in two distinct forms. Type I, the more widespread, is characterized by the appearance of one or more discrete follicles usually situated retrotarsally, but also at any point in the palpebral conjunctiva except over the tarsus. The follicle of this

type is small, hard, shiny, and superficial. It is often transitory, as a rule lasting from 1 to 5 or 6 weeks. There is no other sign of inflammatory reaction in the surrounding tissues. The lesions may be regarded, in accordance with Wilson, who made histological examinations, as a normal hypertrophy of preexisting lymph nodules of the conjunctiva. We have found, as he did, that mechanical manipulation of the lids does not induce generalization, or spread, of follicles and we have failed, like Thygeson, to transfer the affection to fresh monkeys.

In our earlier studies on experimental trachoma, simple folliculosis (Type I) was considered as without positive significance when found to occur in test animals. Olitsky, Knutti, and Tyler (9) state that "a decision as to the positive or negative character of the results was rendered difficult by the mild follicular type of reaction which some of the animals exhibited. . . . Inasmuch as true experimental trachoma is progressive and persistent, only those reactions which endured for long periods of time could, on the basis of our present knowledge, be considered positive."

It would appear, therefore, that this simple condition, which is readily recognizable and clearly differentiated from the other form of folliculosis and from experimental trachoma, need not detain us. On the other hand, Type II, the lesions of which are less commonly met with in nature and which have distinctly progressive and persistent reactions, is worthy of further study and has provided the subject of the present investigation. Therefore, all reference to "folliculosis" henceforth relates to this Type II.

Clinical Appearance.—The essential characteristics of spontaneous folliculosis of Type II are slow progression with infrequent exacerbations; persistence, lasting usually throughout the life of the animal; and marked inflammatory reaction of the conjunctiva.

In a fully developed case occurring in a stock animal, the disease becomes evident only upon eversion of the lids. The upper and lower conjunctivae of both eyes are usually affected. At the inner canthus there may be a drop or two of faintly yellowish, mucoid secretion. The palpebral conjunctiva, including the area over the tarsal plate which, as a rule, is less involved, is congested and appears brownish red. The vascular structures are obliterated and there is considerable edema with thickening of the lid, especially the upper one. The entire surface is covered by large, greyish yellow, gelatinous follicles which

are often agglomerated into irregular masses. When pressed upon with a glass slide, they rupture and expel a greyish white, grumous material consisting chiefly of cellular elements. Occasionally the granulations are dark red and hemorrhagic. In some animals the lesions over the tarsus are smaller and more regular; in others this area appears only roughened and sandy. In well advanced cases the conjunctiva is thickened, the vessels are injected, large discrete and papilliform confluent follicles are present in the palpebral conjunctiva, and the secretion is either diminished or absent. Neither pannus, nor definite visible scars, ulcers, or pits are seen; but in many instances follicles appear on the bulbar conjunctiva.

Histopathology.—The epithelium is thinned out or denuded over most of the surface; but in some areas large goblet cell formation along with thickened epithelium may be seen. A varying degree, but never a marked one, of subepithelial cellular infiltration is visible, consisting chiefly of lymphocytes together with some clasmatocytes and a few plasma cells. The follicle contains an outer zone of lymphocytes, beyond and among which are scattered plasma cells. The central area appears lighter, owing to the closely packed mass of clasmatocytes—cells with vesicular nuclei, without much chromatin, and with fairly clear, homogeneous cytoplasm. The fresher lesions contain in the central area, besides clasmatocytes, a few primitive connective tissue cells and monocytes. Mitotic figures are often present. There are fine bands of connective tissue surrounding the follicles with slender strands entering the latter. The lesion may be considered as a folliculoma rather than a granuloma, for granulation tissue and giant cells are absent.

Bacteriology.—Bacteriological examination of affected conjunctival tissue yields a variety of microorganisms. These will be described in a succeeding paper. At this point, however, we wish to remark that *Bacterium granulosus* (1, 2) has not been encountered in spontaneous folliculosis, although over 50 animals have been studied for the express purpose of isolating this microorganism.¹

¹ We desire to state here that in addition to several indifferent varieties of bacteria we have isolated and cultivated from spontaneous conjunctival folliculosis of monkeys a microorganism capable of reproducing the disease in normal monkeys. The organism in question is a minute, aerobic, actively motile, encapsulated, Gram-negative bacillus, having a single (sometimes double), polar, long flagellum. The cultural and pathogenic properties of this new species of bacterium will be described in a subsequent paper.

Transmission Experiments²

Transmission by Inoculation of Tissue Suspensions.—The conjunctival tissue of monkeys having spontaneous folliculosis was removed, prepared for inoculation, and injected subconjunctivally in the left upper lid of normal *Macacus rhesus* monkeys after the manner of Noguchi (2). The results were uniform in practically all cases.

Within 24 hours after inoculation, marked acute conjunctivitis developed. This was accompanied by pronounced edema and considerable thickening of the lid which extended so as to include 2 or 3 cm. of the cutaneous tissue over the outer canthus and the supraorbital ridge. Abscess formation rarely occurred. After 5 to 7 days the acute process subsided but the eyelid still drooped and the skin pitted on pressure. On eversion, a definite greyish yellow discharge was seen. The upper conjunctiva was markedly roughened, edematous, hyperemic, and purplish red in color. At this early stage large and small follicles were visible, the larger granulations appearing retrotarsally and the smaller ones over the tarsus, and the tortuous course of the preexisting blood vessels could be perceived. The remaining uninoculated conjunctivae exhibited congestion, slight edema, and roughened surfaces. After another week the upper and the two lower uninoculated conjunctivae showed the follicular reaction, while the inoculated lid became more markedly involved. From 14 to 21 days later, all the conjunctival tissue exhibited the clinical and anatomical changes characteristic of the fully developed spontaneous folliculosis observed in stock animals.

The following protocol summarizes the results of the tests.

In the first instance individual suspensions of the conjunctival tissues of seven stock monkeys having folliculosis were inoculated into twenty-four normal animals, two to four serving for each test. The tissue of the seven stock monkeys induced folliculosis in twenty-two of the twenty-four monkeys employed. The material of an eighth affected animal was without effect in either of two monkeys. Thus, including this last mentioned exceptional result, of twenty-six monkeys inoculated with material from eight affected stock animals, twenty-two developed characteristic folliculosis.

The second test consisted of inoculations into normal monkeys of tissues derived from the experimentally induced lesions. By this means the follicular conjunc-

² All operative procedures were carried on with the aid of ether anesthesia.

tivitis was propagated through eight passages to date. The individual suspensions of tissues from thirteen monkeys with experimental folliculosis were injected into twenty-four animals—two for each case except in two instances, in which only one animal was inoculated—and all twenty-four showed marked, characteristic conjunctival folliculosis.

Transmission by Swabbing Secretions on the Conjunctiva.—

In still another experiment, a cotton swab was applied to the lesions of an affected stock monkey and the secretion thus secured transferred directly, by gentle rubbing, to the smooth upper left conjunctivae of two monkeys. After six such swabbings, over a period of 6 days, folliculosis was manifest in both animals: the treated eye revealed first signs of the affection on the 3rd day after the last swabbing; the untreated, on the 10th day.

In summarizing these results, we find that only one of twenty-two animals having folliculosis yielded material not capable of producing the disease, and that of 52 monkeys inoculated with suspensions, or swabbed with the secretions, of affected conjunctival tissues, forty-eight showed signs of folliculosis indistinguishable from those seen in nature. Hence it appears that the *Macacus rhesus* monkey is highly sensitive to the active agent of the disease. The inference is that folliculosis is infectious, a view supported by the results of the contact experiments to be described.

Control Experiments.—Olitsky, Knutti, and Tyler (1, 9–12), Finnoff and Thygeson (13), and others have reported numerous control tests on monkeys in which different materials, such as human and monkey conjunctival tissue, culture media, saline solution, and various species of bacteria, have been injected subconjunctivally with uniformly negative results. We have observed that mere scarification of smooth, normal conjunctivae has not been followed by folliculosis.

During the course of our studies we inoculated twenty-one monkeys with bacteria encountered irregularly in cultures derived from folliculosis tissues, consisting chiefly of Gram-negative, chromogenic bacilli, but without specific effect; and we injected seven monkeys with microorganisms obtained from human non-trachomatous conjunctivitis, likewise with non-specific results. Cultures of three organisms secured from cases of trachoma in Tunis and sent us by Dr. Thygeson were also without effect in nine monkeys.

Suspensions of normal monkey conjunctival tissue were inactive in ten animals and Berkefeld V filtrates of similar materials were non-pathogenic in four.

Four monkeys injected with saline solution washings of sterile agar slants, and

two swabbed conjunctivally for 5 consecutive days with material from cages in which they were confined failed to show folliculosis.

From the results obtained in the 57 monkeys employed in this series of tests, it would appear that the disease is not set up in normal conjunctivae by the injection of various control materials. The effects of inoculating tissue suspensions derived from cases of spontaneous folliculosis would appear to be specific.

Contact Experiments.—In view of the fact that selected monkeys with smooth conjunctivae kept in quarantine did not show folliculosis, and did not develop the disease after injection of non-folliculosis materials, the following positive results of contact experiments take on significance.

Three experiments were made. In each, two monkeys having smooth conjunctivae were caged with two animals showing folliculosis. 2 to 10 weeks later the six test animals that had been previously normal exhibited characteristic follicular conjunctivitis in both eyes.

We have already commented on the fact that the disease can be spread by contact from the inoculated conjunctiva to the uninoculated membranes of the same monkey. From the foregoing experiments it is clear that spontaneous folliculosis can be conveyed from affected to normal animals by simply caging the two together.

The fact that the disease can be transmitted to normal animals by contact brings out the difficulties of maintaining quarantine, especially when monkeys are handled during experimentation. That we succeeded in overcoming these difficulties is demonstrated by the small number of incidental follicular infections that developed, namely, four in a group of 250 animals. These were observed during the early stages of the investigations after the animals had been released from quarantine.

Filtration Experiments

We next attempted to determine whether the active agent of spontaneous folliculosis will pass through filters that retain ordinary bacteria.

Ten separate filtration tests were made with Berkefeld V filters and other more permeable filters of the same sort, designated in Table I as "VV," which permitted

the passage of *B. prodigiosus* (14). In further tests Seitz single disc filters were also used. The material for filtration consisted of fragments of excised conjunctival tissue, removed from monkeys having fully developed spontaneous folliculosis, suspended by grinding in either physiological saline solution or hormone broth. As a rule, fragments of the conjunctivae of four lids were ground in 5 to 10 cc. of the suspension fluid and, after sedimentation of the larger particles of tissue, the faintly clouded supernatant fluid was transferred to the filtering apparatus. Filtration was made at a negative pressure of 60 cm. Hg and continued until the entire suspension had passed into the filter candle or disc—the

TABLE I
Results of Filtration Tests

No. of experiment	Type of filter used	Fluid used for suspension	Results of inoculation of monkeys			
			With unfiltered material		With filtered material	
			No. inoculated	No. showing folliculosis	No. inoculated	No. showing folliculosis
1	Berkefeld V	Saline solution	2	2	3	0
2	"	" "	2	2	3	0
3	{ " V " VV " V " VV " V	" "	2	2	{2 2	{0 0
4		" "	2	2	4	0
5		" "	2	2	2	0
6		" "	2	2	2	0
7		Hormone broth	2	2	3	0
8	"	"	2	2	3	0
9	Seitz	"	1	1	{2 2	{0 0
		{Saline solution				
		{Hormone broth				
10	"	{Saline solution	1	1	{2 2	{0 0

procedure requiring usually less than 10 minutes. We hoped that by these means the infectious agent passing into the filtrate might be not too greatly diluted for pathogenicity. The results of these tests are summarized in Table I.

A summary (Table I) of the results of filtration experiments on 50 monkeys indicates that all of eighteen animals inoculated with unfiltered folliculosis material developed characteristic folliculosis. On the other hand, none of thirty-two monkeys injected with filtrates obtained in various ways, including those secured from very permeable filters, showed the disease. The active agent failed to pass even through filters that let through *B. prodigiosus*.

Absence of Inclusion Bodies.—Many filtrable viruses have distinctive cytotropic effects (15) and cellular inclusion bodies are characteristically associated with the action of some of them. As the following protocol shows, no such bodies have been found in the cells derived from spontaneous folliculosis cases.

Film preparations were made from fourteen monkeys showing early folliculosis. Lesions within 4 weeks after their onset were examined because the so called inclusion bodies of granular conjunctivitis of various types are found most often in the first stages of the disease. Films stained by the method of Giemsa, from one to three slides in each case, were examined with the aid of a mechanical stage, the entire film being searched. No inclusion bodies were found,³ nor were such bodies discoverable in any of the large number of histopathological sections of affected conjunctival tissues, stained by Giemsa's method and by eosin-methylene blue.

CONCLUSIONS

Spontaneous folliculosis of *Macacus rhesus* monkeys—a type of follicular conjunctivitis associated with marked, local, inflammatory reactions—is apparently a disease *sui generis*, due to a specific infectious agent. It can be transmitted from monkey to monkey by means of subconjunctival injection of suspensions, and by conjunctival swabbing of the secretions, of affected tissues, or by contact of normal animals with folliculosis monkeys.

The agent causing folliculosis has failed in our hands to pass through Berkefeld and Seitz filters, even those of an unusual degree of permeability; and the lesions that it causes show no cellular inclusions suggestive of the action of a virus. The condition is due apparently to an organism of low grade pathogenicity. The essential histopathological structure corresponds to that of a folliculoma (16) which, while not identical with a granuloma, bears certain resemblances to the latter.

The studies here reported concern only one species of monkey, *Macacus rhesus*. Further investigations will be carried out on different species of Anthropeoidea and other animals.

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³ We are indebted to Dr. R. E. Knutti for his examination of the slides from five of the monkeys.

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BIOLOGICAL STUDIES OF THE TUBERCLE BACILLUS

II. A NEW CONCEPTION OF THE PATHOLOGY OF EXPERIMENTAL AVIAN TUBERCULOSIS WITH SPECIAL REFERENCE TO THE DISEASE PRODUCED BY DISSOCIATED VARIANTS

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PLATES 19 TO 24

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Our knowledge of bacterial variations existing within a definite group has been greatly enriched in the last 10 years by the studies of men who have refused to accept the dogma of monomorphism and stability. It has become more and more evident that bacteria of very widely separated genera, and in pure line of culture originating from a single cell, may break up into two or more different strains with characteristics clearly differentiating them one from another. These characteristics may manifest themselves in individual cell morphology, in topography of the colonies, in virulence and in a number of other biological and physical characteristics. Recently one of the present authors (Petroff, with Steenken (1)), reported studies pointing to the fact that the tubercle bacillus followed the same line of variability as other microorganisms. From bovine, BCG and avian stock cultures of tubercle bacilli they separated variants with characteristics similar to those of other dissociated microorganisms.

The organism most extensively studied by the above mentioned investigators was the avian tubercle bacillus A₁. From the original culture, which has been in the laboratory for the last 20 years, they dissociated three distinct types of variants: (1) a small half moth-ball colony, (2) a flat, spreading, slightly wrinkled colony, (3) a large, dry, wrinkled colony.

In the present study we have attempted to describe the primary types of response as they occurred in chickens experimentally infected with dissociated avian tubercle bacilli.

EXPERIMENTAL DATA

In a preliminary study it was found that the dissociated variants from avian tubercle bacilli were much more stable than the variants of either human or bovine cultures. This was true, however, only to a limited degree. To maintain stability, all variants were cultivated on gentian violet egg media with the exception of R which was cultivated on glycerine agar media to maintain avirulence. At first three distinct variants were dissociated with the topographical characteristics above described, namely:

S, Smooth (Figs. 1 and 3).—On gentian violet egg media these organisms developed sparingly and slowly, forming colonies appearing like half moth-balls, the surfaces of which were smooth and moist. They were emulsified easily in salines. On fluid media the organisms propagated in the bottom of the flask forming within a week a diffused and even suspension. With the passing of time a very thin filament appeared on the surface of the fluid.

F. S., Flat Smooth (Figs. 4 and 5).—The colonies of this variant on gentian violet egg media were slightly wrinkled, moist, spreading and from four to six times larger than the S. The organisms were emulsified with difficulty in saline. On fluid media the growth developed best on the surface; at first from a veil-like filament which, as the growth accumulated, became a heavy, wrinkled pellicle. On all media the growth was very profuse and rapid.

R, Rough (Figs. 6, 7 and 8).—On gentian violet egg media the colonies appeared large, wrinkled and perfectly dry. On fluid media they also propagated only on the surface. The rate of growth was very rapid and in a few weeks a small inoculum covered the whole surface of the flask with new growth. It was almost impossible to make a suspension of this variant to consist of individual organisms.

The Fourth Variant, Ch (Figs. 2 and 3).—This was dissociated from the smooth S colonies which were accidentally subjected to a high temperature. The colonies were smooth, half moth-balls in appearance, had all the physical properties of the conventional S with the exception that they became chromogenic and ocher in color and were not pathogenic for chickens. The bacteriological details of this variant will appear in a special study.

Media.—The above four variants were cultivated on gentian violet egg 4 per cent glycerine agar and Proskauer and Beck's media. Young cultures not more than 6 weeks old were used. Colonies of definite topographical structure, corresponding to the above description, were removed from the flask and triturated in a sterile mortar with sterile saline. The suspension was either counted and the number of organisms per cc. determined, or the strength of the suspension determined by weight. All inoculations were made intravenously into the wing veins.

Sixty-one young fowls were used in the experiment, all practically of the same size and age. They were kept under observation for a few weeks and then tested by injecting into the ear wattle, 50 per cent avian tuberculin in saline and the reading was made at the 24th hour.

The blood changes were followed in each case. The Forkner (2) method proved

satisfactory for determining the total leucocyte count and his descriptions were used in conjunction with the supravital method for differentiating the various types of white blood cells. To establish the normal base line several counts were made previous to infection. Once a base line was established for each fowl we experienced no difficulty in following the changing phases of the leucocytic picture after infection. It seems necessary here to call the reader's attention to the fact that in chickens the cells possessing acidophilic staining granules, the so called eosinophiles or pseudoeosinophiles of Maximow, are the predominating granulocytes and correspond to the polymorphonuclear neutrophiles present in human blood in their percentage and function.

Chickens that died and those that were killed by intravenous injection of air, were necropsied as soon as possible. Sections of the various organs were fixed in Zenker's solution and in 10 per cent neutral formalin, imbedded in paraffin, cut at 5 micra and the Zenker-fixed tissues stained routinely with Mallory's phloxin-methylene blue. Direct smears were made from spleen, bone marrow, liver and occasionally from other organs and stained for tubercle bacilli. Small portions of the above mentioned organs were also inoculated on gentian violet egg media. In some cases tissue smears were stained supravitaly to facilitate the identification of particular cells. To demonstrate acid-fast organisms sections were stained either by Ziehl-Neelsen or Cooper's modification, followed by an appropriate counterstain. For this purpose a hematoxylin, azur-orange G counterstain was useful in differentiating the bacilli from the granules of the eosinophiles. Gram-Weigert's stain was used for fibrin and bacteria. Collagen was studied either by Van Gieson's or by Mallory's aniline blue staining method. Foot and Foot's (3) modification of Bielschowsky's silver impregnation method was used to demonstrate the reaction of the reticulum to the tubercle formation. In studying the lesions, comparisons were made between identical tissues of different birds in order to control variations that might occur due to local or organ resistance and anatomical situation.

Smooth S Variant

Nineteen chickens were inoculated intravenously with freshly prepared saline suspensions, four receiving 5,000, four, 5,000,000, seven, 0.16 mg., two 0.25 mg., two, 0.33 mg. of these bacilli. Following the inoculation, blood studies were made at the 18th, 24th, 48th, 114th hours and at weekly intervals.

A number of the chickens died of tuberculosis during the period of observation (one each on the 5th, 14th, 26th, 27th, 28th, 29th, 31st, 41st, 43rd and 110th days). Others were killed at intervals to determine the progress of the disease (two on the 7th, one on the 21st, two on the 35th, one each on the 37th, 51st and three on the 78th days).

The blood picture (Chart 1) is of considerable interest. The eosinophiles responded early by beginning to rise during the first few days and continued ascending, running more or less parallel with the total white blood cell count. During

the eosinophilia a slow rise of monocytes occurred with a decrease of lymphocytes. These lines usually crossed between the 1st and 2nd weeks, the lymphocytes dropping occasionally to the extremely low level of 5 to 0 per cent.

Pathological findings revealed evidence of an acute infection of malignant type, coupled with "toxic" manifestations. Macroscopic tubercles were generally absent in the early stages. They appeared at about the end of the 4th week as a very fine, white stippling of the serosal surface of the liver and spleen. The most important finding in the gross examination of the viscera was the marked enlargement of the spleen and liver which occurred as early as the 3rd week. At this time these organs were usually swollen to three or four times their normal size, yellowish red in color and of soft consistence. The bone marrow often appeared red and increased in amount, a functional hyperplasia to meet the greater demand for blood cell production.

Microscopic lesions were present in the liver of all but two cases, in the spleens of all but six, in the lungs of only six and even less often in the bone marrow, kidneys and intestines. There was a direct relationship between the dosage and the extent of the disease. In those inoculated with 5,000 and 5,000,000 bacilli the disease was present in the liver of three out of four, and in the spleen of only one out of four cases. The smaller dosage had a tendency to establish a chronic disease. Extension to the lungs occurred only when the infection was made with 0.16 mg. or more. The bone marrow was involved occasionally and then only when at least 0.33 mg. was used.

Microscopic examination revealed tubercles of an acute exudative nature. After the 2nd week these lesions were of irregular shape and composed of clumps of proliferating, large mononuclear cells with little or no peripheral zone of lymphocytes. In the liver they appeared to be actively invading the surrounding parenchyma with the adjacent liver cells undergoing rapid dissolution (Fig. 13). During the 3rd and 4th weeks the beginning of abscess formation took place at the center of these tubercles. Giant cells were absent unless there was considerable caseation. Occasional deposits of fibrin were found in the centers of the lesions. There was fatty infiltration of the liver cells at some distance from the tubercles. Acid-fast bacilli were present within the lesions but usually very few in number (Fig. 25). Smears and cultures from the spleen, bone marrow and occasionally liver, were usually positive. In three chickens receiving a 5,000 bacilli dose they were negative. The recovered cultures were of the same type as those inoculated.

Subinoculations.—Two chickens in the main group, which had been inoculated with 0.16 mg. of a suspension of S organisms containing only rods, ran a course of disease typical of this type. This, however, was true only as far as the blood picture and the tubercle formation were concerned and not of the morphology and distribution of the bacilli. One chicken developed leucocytosis and an increase of eosinophiles and was killed on the 37th day. The spleen and the liver were about three times their normal size. Microscopic examination disclosed exudative S type of tubercle formation in the liver, spleen and lungs. Direct smears from the tissues

revealed only branching forms of acid-fast bacilli (Figs. 30 and 31). Small portions of the spleen and bone marrow were triturated separately with saline and inoculated intravenously into three healthy chickens: two with the spleen and one with the bone marrow suspensions.

Of the two chickens inoculated with the splenic suspension, one became sick at the end of 40 days and was killed. The other lived 58 days before becoming ill and was also killed. The blood counts of both showed an eosinophilia, increasing steadily from week to week, along with a decrease of lymphocytes, and a rising total white blood cell count. There was also a monocytosis which increased at the expense of the lymphocytes. The total counts rose to 133,150 and 152,040. The chicken inoculated with the bone marrow, apart from a slight temporary monocytosis and eosinophilia, showed no significant blood change. Necropsies showed enlargement of the liver and spleen three times the normal size and studded with small white nodules. Smears from the liver, spleen and marrow were positive for acid-fast rods, with no evidence of branching forms. Cultures from all tissues revealed colonies of white S topography which were made up of typical rod-shaped bacilli. Microscopic examination of the tissues revealed large, single and conglomerate S tubercle formation with early necrosis in one chicken and very extensive necrosis in the other. Similar lesions were present in the spleen, liver, lung and marrow and also in the kidney of the latter case. The chicken which had been inoculated with the bone marrow suspension was negative and microscopically disclosed no evidence of tuberculosis. Smears and cultures were likewise negative.

Another chicken of the main group became moribund and was killed on the 43rd day. It had shown an increasing eosinophilia reaching as high as 63 per cent before being killed. Direct smears from the spleen and marrow were negative for acid-fast organisms. The cultures from the liver and spleen, however, developed a growth of typical S variant. At necropsy, pieces of liver, spleen and bone marrow were triturated with normal saline and inoculated intravenously into three normal chickens, one with 5 cc. of liver, another with 4 cc. of spleen and a third with 2 cc. of bone marrow suspensions.

The fowl inoculated with the liver preparation showed little blood reaction until after 4 weeks, when an eosinophilia and monocytosis took place with a total leucocytosis of 118,570. The eosinophilia continued until the 62nd day, when the chicken died. At necropsy the peritoneal cavity was filled with bloody fluid and a fresh laceration 1 cm. in length was found in the liver which was swollen to three times its usual size, of soft consistence and of yellowish red color. This rupture and hemorrhage had occurred, perhaps following the slight trauma, due to the extremely soft consistence of the swollen tissue. The spleen was of pale red color and enlarged to three times its average size. Macroscopically, both organs showed numerous, gray, miliary nodules on the serosal surface. Direct smears from these tissues and the bone marrow were positive for acid-fast rods. All cultures were positive with typical white S colonies. Microscopic study disclosed the nodules to be large tubercles many of conglomerate formation with prominent centers of necrosis

lined by a margin of giant cells, some of which showed degeneration and necrosis. Similar lesions were present in the lungs and bone marrow; in the latter tissue was also extensive caseation and necrosis. The younger non-caseating tubercles were of acute, exudative S nature. There was marked necrosis of the liver cells, some of which showed invasion by endothelial leucocytes.

As the chicken inoculated with spleen suspension and killed on the 54th day revealed the same pathology and blood picture as the preceding bird, the description will be omitted.

The chicken inoculated with 2 cc. of bone marrow suspension was killed at the end of the 84th day as it had shown no indication of infection by the blood picture. There was no gross or microscopic evidence of tuberculosis and smears and cultures from the spleen and bone marrow were negative for tubercle bacilli.

Flat Smooth F.S. Variant

Twelve healthy young chickens were inoculated intravenously with freshly prepared saline suspensions of this variant. Two received 5,000, two, 5,000,000, four, 0.16 mg., two, 0.25 mg., and two, 0.33 mg. of bacilli. Following the inoculation the blood counts were made at the same intervals as described in the preceding group.

Many of the chickens of this group also succumbed to tuberculosis during the period of observation. Deaths occurred on the following days: 20th, 28th, 33rd, 39th, 41st and 45th. Because of their moribund condition a few were killed on the following days: 21st, 31st, 46th and 69th. Two were killed to examine the disease process on the 13th and 35th days. There was some correlation between the dose of infecting bacilli and duration of life. Inoculation with 0.33 mg. of bacilli killed one in 20 days and 0.16 mg. killed in 33, 39 and 41 days. A dose of 5,000 bacilli was not fatal even at the 69th day.

The differential leucocyte counts indicated a virulent type of infection leading to rapidly progressing disease (Chart 2). The eosinophiles showed no appreciable reaction until the disease had approached a terminal stage. At this time they increased rapidly, accompanied by a leucocytosis which was maintained until death. During the course of the infection, some time before the terminal stage was reached, the monocytes began to increase in numbers at the expense of the lymphocytes, this occurred along with the eosinophilia. The total white cell count rose slowly with the monocytosis, but did not begin to ascend rapidly until the eosinophiles had definitely assumed a prominent part and the disease had reached its final stage.

Pathological findings agreed with the blood reactions and we found extensive tubercle formation due to rapidly multiplying bacilli. As few as 5,000 bacilli produced infection in the spleen and liver and as the dose was increased there was marked extension of the disease throughout the viscera. Small tubercles became visible grossly in the spleen and liver 20 and 21 days after inoculation; from the 3rd week on these two organs also began to increase somewhat in size. A few microscopic tubercles were present in the spleen and liver of a chicken infected with 5,000

bacilli after the 35th day. The disease in another, which received a similar dose, had extended as far as the lungs after 69 days. Five million organisms produced lesions in the spleen, liver, lungs and bone marrow in two chickens after 28 and 46 days, respectively. As the dose was increased the lesions were found not only in the spleen, liver, lung and bone marrow, but often in the intestine and heart muscle. This took place as early as the 21st day. Smears and cultures from the spleen and bone marrow revealed acid-fast organisms in all fowls of this group with the exception of two inoculated with 5,000 bacilli where only cultures and not smears of the bone marrow, were positive.

Microscopically, the tubercles were composed of rounded clumps of closely packed epithelioid cells containing many phagocytized bacilli, which often lay together in large masses sometimes showing a radial arrangement (Fig. 26). About the periphery of the lesion there was usually an exudate of lymphocytic and plasma cells which surrounded a definite zone of hyalin material (Fig. 14). Although this material had somewhat the appearance of fibrin, special staining methods demonstrated its collagenous nature. Usually after the 3rd week there was evidence of necrosis and abscess formation which progressed rapidly to extensive caseation and production of numerous giant cells about the caseous areas. With the extension of the tubercle the peripheral hyalin zone tended to break apart and disappear, permitting dissemination of the bacilli into uninvolved tissue with the formation of both new and conglomerate tubercles.

Rough R Variant

Eight healthy young chickens received freshly prepared saline suspensions of this organism intravenously. Two received 5,000, two, 5,000,000, and four, 0.25 mg. of bacilli. Blood counts were done at the usual intervals to follow the reaction of the blood leucocytes.

None of the chickens in this group died during the time of observation. Two were killed on the 7th day; two on the 21st; one on the 50th; two on the 78th, and one on the 79th day.

Infection by this variant produced a leucocytic reaction, differing from the two preceding groups (Chart 3). By the 24th hour count there was usually a complete return to normal of the early eosinophilia. From then on lymphocytes increased although in most cases they were exceeded by a monocytosis which took place anytime during the 2nd to 6th weeks. In some this monocytosis was marked, accompanied by a leucocytosis of 107,550 in one instance, and 152,120 in another. Previous to this the lymphocyte and monocyte lines occasionally crossed each other but usually by the 8th to the 11th weeks the lymphocytes had again become the predominant cells. The eosinophiles did not take an active part in the leucocytic reaction after their early initial rise and fall. In four chickens, after 11 weeks, the differential and the total leucocyte counts had returned to the normal level existing previous to infection with the exception of a slight monocytic elevation. Such a leucocyte picture occurred in all chickens inoculated with this variant no matter how heavy the dosage.

Gross examination of the viscera was negative approximately 11 weeks after infection in four chickens. Microscopically, tuberculous lesions were found in the spleen and liver of these birds and in the lungs of two, one killed 50 days after inoculation with a 0.25 mg. dose of bacilli, and the other 78 days after a similar dose. Direct smears from the spleen were positive for bacilli in six chickens and positive cultures were recovered from the spleens of three. Organisms were never demonstrated in the bone marrow either by smears or cultures. Bacilli were found oftener in the chickens killed by the 21st day; in three which were allowed to live for 75 days or longer, no organisms could be recovered. This speaks for their rather early destruction. The microscopic lesions were small in size and of retrogressive or stationary nature. In the liver especially, these lesions were no larger than two or three hepatic cells. They consisted only of a few large mononuclear and epithelioid cells, often surrounded by a fringe of lymphocytes (Figs. 16 and 24). There was never any evidence of caseation or abscess formation, because there was insufficient bacillary growth or derivatives to cause an influx of eosinophiles leading to necrosis. Such tubercles, at the 11th week, had not advanced to a stage comparable to that reached by the S or F.S. tubercles during the 1st week. Occasionally, instead of tubercle formation, we could find an isolated cluster of giant cells showing no evidence of inflammatory reaction. According to Medlar's (4) interpretation this is a healing process in itself. In one chicken which had been inoculated with a 0.25 mg. dose and killed on the 79th day, microscopic study of sections from liver, spleen, bone marrow, lung, kidney, heart, adrenal and testis was negative for tubercle formation. Prolonged search in sections stained for tubercle bacilli disclosed in only one instance a single acid-fast rod lying in a subsinusoidal area of the liver, probably within the cytoplasm of a phagocytic cell, although this could not be ascertained. There was no cellular or exudative reaction resembling tubercle formation set up by the presence of this organism. Yet smears from the spleen and liver were positive for acid-fast bacilli and the sediment and supernatant fat layer from a sodium hydroxide digest of these organs gave abundant growth of typical R colonies on gentian violet egg media. Portions of this digest, used for inoculating the plates, were injected into a chicken intravenously and intraperitoneally. It showed a non-septic leucocytic reaction and was killed after 75 days. Microscopic tubercles of the R type were found in the spleen and liver. Direct smears from these tissues were negative for acid-fast bacilli, but the culture from the spleen was positive. Therefore, the viability of the organisms in the tissues of the chicken was established by production of lesions and cultures.

It was impossible to demonstrate the presence of bacilli in this benign type of tubercle formation by our ordinary methods. They are present, but so few that only careful study of serial sections of such a lesion could reveal them.

Rare tubercles were found in the liver of birds inoculated with 5,000 and 5,000,000 organisms as early as 7 days. Cultures and smears from the spleen were positive for acid-fast bacilli in both instances. Two 21 day chickens were microscopically negative for tubercle formation but direct smears from the spleen were positive.

Chromogenic Ch Variant

2

Fourteen chickens received freshly prepared saline suspensions of this variant; four, 5,000; four, 5,000,000; three, 0.25 mg.; two, 0.33 mg., and one, 0.50 mg. of bacilli. The reaction of the white blood cells was followed as in previous groups. None of the chickens in this group died during the periods of observation. Two were killed on the 7th day; two on the 21st; one on the 31st; two on the 35th; one on the 47th; one on the 78th; four on the 80th, and one on the 93rd day. A saline suspension prepared from the liver and spleen of one fowl, killed on the 31st day, was subinoculated intravenously and intraperitoneally into a healthy chicken to demonstrate the authenticity of the lesions. This subinoculated chicken was killed after 54 days.

The reaction of the blood leucocytes, with slight variations, was definitely characteristic (Chart 4). The early 18th hour non-specific eosinophilia tended to vary directly with the dosage used and subsided by the 42nd hour. The total leucocyte count would often show a slight, prolonged rise, starting after the first few days and maintained for most of the period of observation. During the 3rd to 6th weeks a temporary monocytosis would occur. This would recede after a varying length of time, and the lymphocytes again regain their superior level where they remained until the end of the period of observation. Usually by the 75th day the entire white blood cell picture had returned quite closely to the normal level existing previous to infection except for some elevation of the monocytes.

Pathological findings point conclusively to the relatively benign nature of the infection established by this variant. The blood reaction indicates a successful defense on the part of the host as these organisms of low grade virulence are dealt with and overcome. Almost all of the bacilli seem to have been destroyed, regardless of how heavy the dose. Smears and cultures obtained at necropsy were not able for their paucity of acid-fast organisms or growth. 5,000 bacilli produced no infection in four chickens up to 80 days and the bacilli could not be recovered. Of four birds inoculated with 5,000,000 organisms, tubercles were found in the spleen and liver of one after the 35th day, and from these lesions organisms were demonstrated by smears and cultures. A second chicken, killed on the 21st day, and a third on the 80th day, were entirely negative for tuberculosis both microscopically and by culture. From the fourth, killed on the 7th day, a positive culture was obtained from the spleen, yet microscopic examination of sections failed to disclose any tubercle formation. A larger dose, 0.25 mg. of bacilli, resulted in tubercle formation in the spleen and liver in two chickens after 47 and 78 days, respectively. In the latter, only the bone marrow culture was positive while in the former both spleen and liver contained acid-fast bacilli by smear and culture. Two chickens, each inoculated with 0.33 mg. of a culture which had been passed through chickens, showed no tubercle formation or other evidence of infection when killed on the 80th day, with the exception of a few typical Ch colonies on a plate inoculated with a suspension of bone marrow. It seems that the virulence of this variant is not enhanced by a single bird passage. In this entire group the disease, when it occurred,

was apparently limited to the liver and spleen. Lungs, bone marrow, kidney and intestine escaped involvement. As might be expected in a disease of such limited character, tubercle formation consisted only of minute clumps of large mononuclear and epithelioid cells, usually infiltrated by a few lymphocytes and possessing a peripheral zone of lymphocytic cells (Figs. 15 and 23). There was never any evidence of necrosis or abscess formation.

In none of the sections were we able to demonstrate bacilli within the tubercle. However, the viability of the organisms in the lesions was proved by subinoculation of tissues into a healthy chicken, which reproduced lesions in the second bird, and also direct smears revealed the presence of acid-fast bacilli.

The Reaction of the Blood Leucocytes

Study of the peripheral blood, employing the supravital stain for the differential leucocyte count and Forkner's total white blood cell determination enabled us to closely follow the course of infection. Freely flowing blood was taken from the wing vein and the preparations studied within a few minutes. The same person did all the differential counts and another the total white blood cell counts and the two estimations were determined from the same blood sample.

We have not attempted to consider the very early reactions which begin upon entrance of the bacilli and result in their localization in certain phagocytes as this has been well described in the studies of Vorwald (5) and Long (6). From somewhat limited observations we believe that this process may be the same no matter which variant is used.

Chart 1 illustrates the fluctuations of the average total and differential white blood cell counts in five chickens infected with S variant. It shows clearly the relatively acute course of the disease, and the extent of the leucocytosis which begins after the 48th hour, ascending steadily until the last days of life. In the differential count the increase in the percentage of eosinophiles stands out in contrast to the rapid fall of lymphocytes. Monocytes show an upward trend which crosses the lymphocyte line between the 10th and 15th days. This type of blood reaction is characteristic of this variant. It suggests a rapidly progressive disease of an acute "toxic" nature and a fatal termination.

Chart 2 represents the averages of the blood leucocytes of five chickens inoculated with the flat smooth F.S. variant. The curves suggest a virulent, rapidly progressive disease somewhat similar to that produced by the S. The eosinophiles, however, do not react with the same speed as in the S type and the rise occurs only when the disease attains its fatal termination. The total leucocyte count also ascends but is much slower in its rise than in the S. Here the disease requires a longer time before it acquires the momentum necessary to produce the profound leucocytic reaction seen in the S infection. Monocytes and lymphocytes show approximately the same type of curve as in Chart 1, the former rising and the latter falling as the disease progresses.

Chart 3 illustrates the average blood leucocyte count in five chickens inoculated with R variant. The points worthy of notice are the failure of reaction on the part of the eosinophiles and the supremacy of the monocytes over lymphocytes

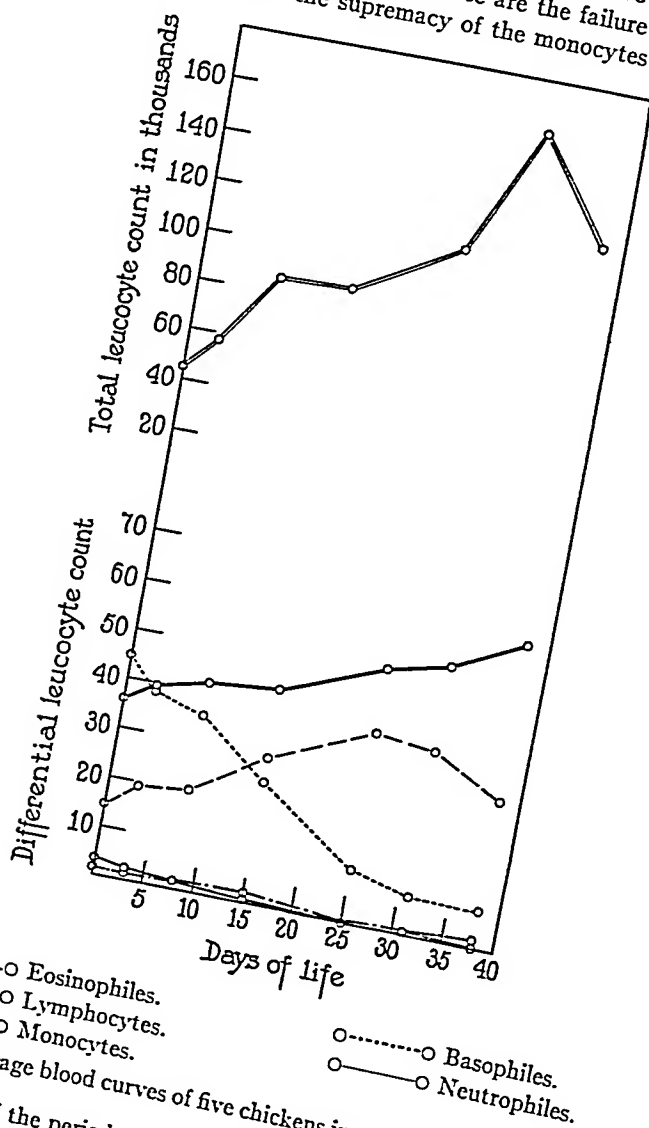


CHART 1. Average blood curves of five chickens inoculated with S variant.

until the last half of the period of observation when the lymphocytes again cross the monocyte line and approach their normal level. The rise in total leucocyte count was maximum about the 5th to 6th weeks and then receded to approximately

normal. This leucocyte reaction suggests rather conclusively a tuberculosis of relatively benign type. However, we cannot definitely state that there was recovery from the infection, as the period of observation was limited to 77 days.

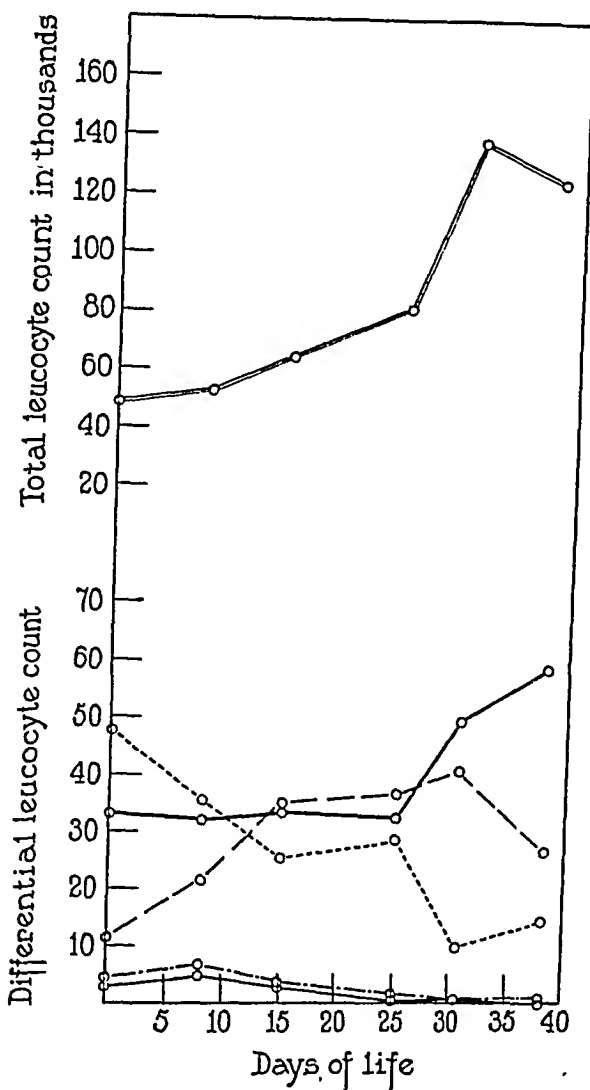


CHART 2. Average blood curves of five chickens inoculated with F. S. variant.

Chart 4 depicts the average of the blood reactions in five chickens infected with chromogenic Ch variant. The period of study (73 days) does not mark the end of the disease. Usually after the 10th week the differential and total white blood cell count had returned to the normal level observed prior to infection with the exception of a slight monocytosis. It certainly reveals a marked resistance of the host to a relatively avirulent variant. The lymphocytes, ascending to a high level,

remained there for a considerable length of time. From the 4th to the 6th weeks they were displaced by monocytes which eventually gave way to a return of the lymphocytes. At no time was there eosinophilia, but rather a failure of response on the part of these cells. An increase of the total leucocyte count occurred during

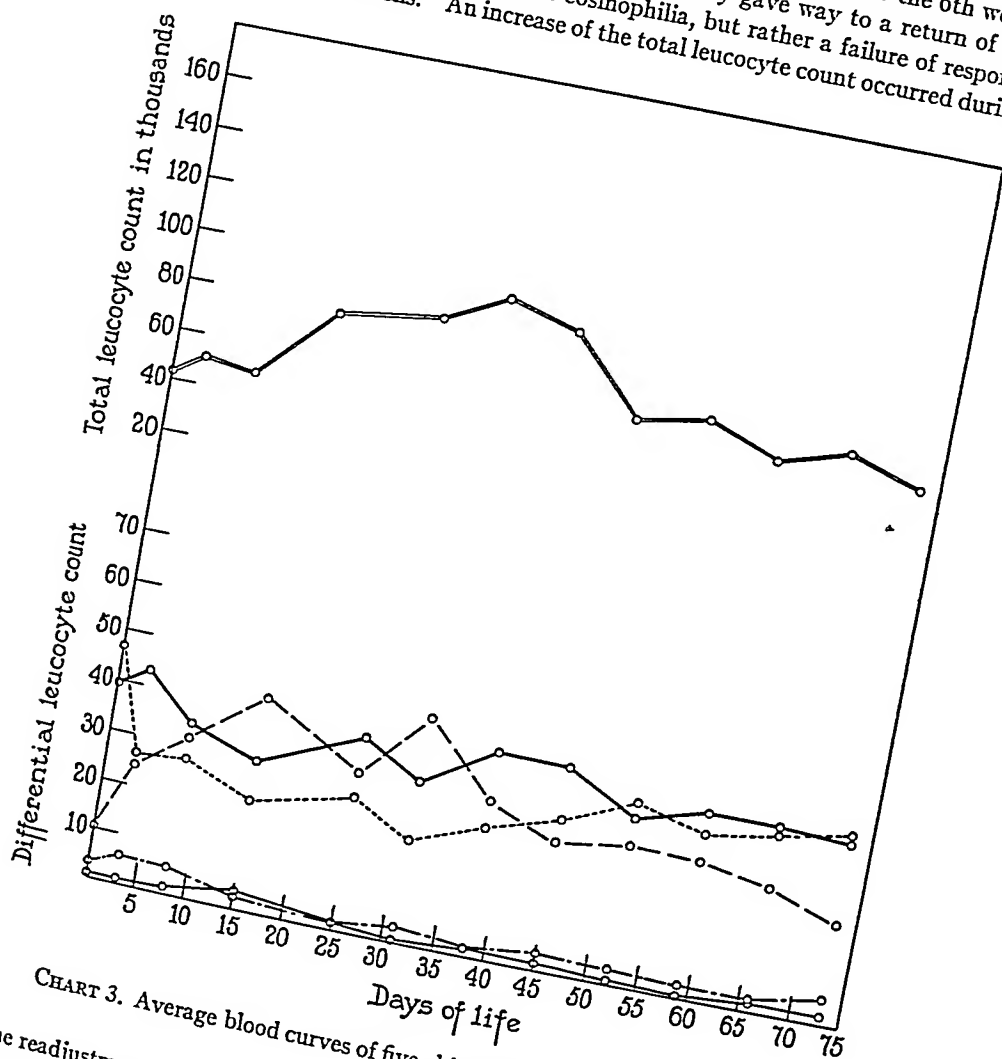


CHART 3. Average blood curves of five chickens inoculated with R variant.

the readjustment on the part of the monocytes and lymphocytes but never to any great extent. From the appearance of the leucocytic reaction alone, one can say that this is also a relatively benign form of avian tuberculosis and that the blood reaction is a direct reflection of the pathological process.

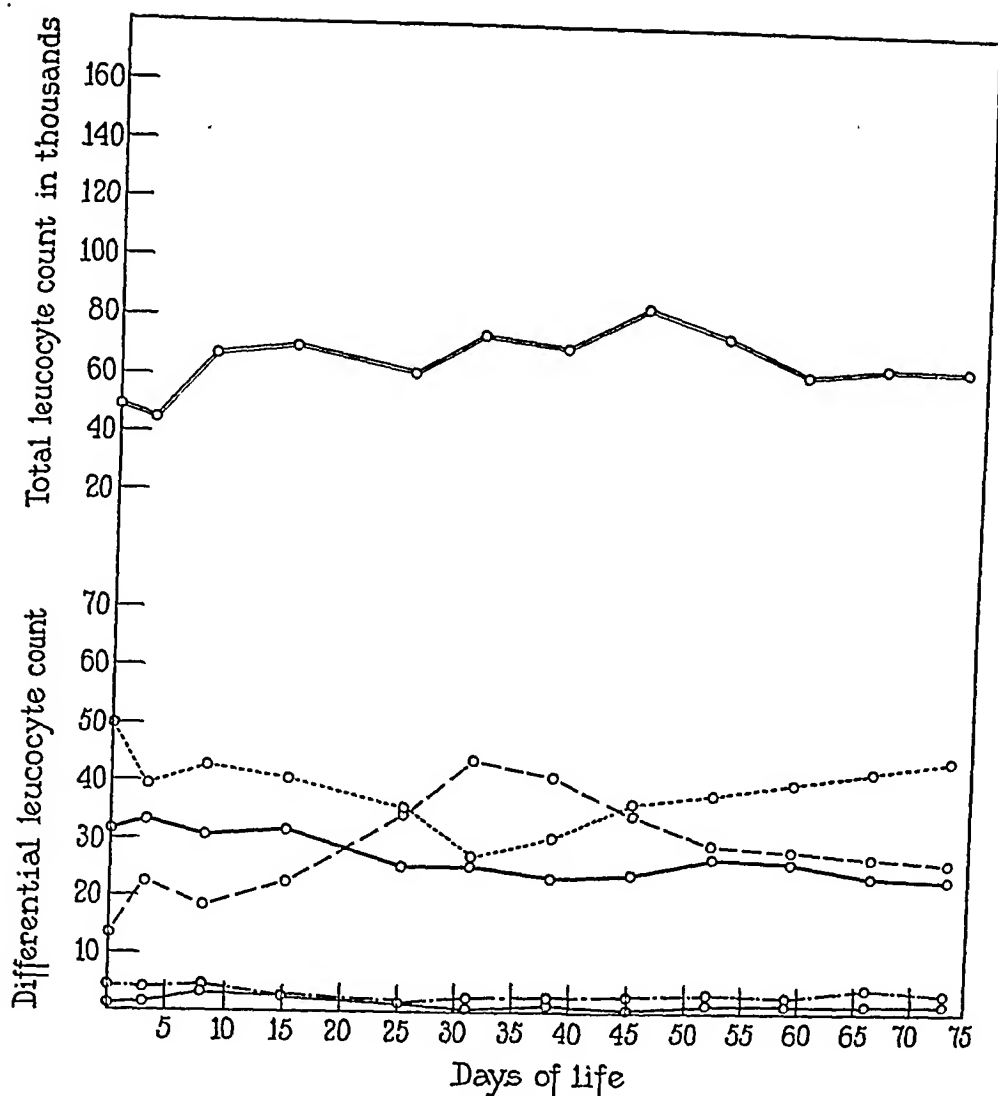


CHART 4. Average blood curves of five chickens inoculated with Ch variant.

Pathogenesis

An early non-specific eosinophile increase (polynucleosis) was the first reaction following the entry of the organism into the blood stream. It was non-specific because it occurred in all birds inoculated with the four dissociated variants. This rise usually subsided after the 18th hour. At this time, as has been previously observed in the lungs of rabbits by Vorwald and Long, the infection became localized in the tissues by aggregation and clumping of bacilli-laden eosinophiles (Fig. 9). The blood changes reported in these studies do not include this early non-specific reaction for the reason that not until it had subsided did the blood leucocytes begin to show the variations apparently related to the type of variant used for infection.

Following this early reaction, in chickens infected with smooth S variants, eosinophiles increased very rapidly. Such a change is lacking in the other type. The blood picture was typical of that seen in acute infectious diseases and, as we shall see later, was probably not due to the number of organisms present in the lesions but very likely to "toxic" substances liberated by the bacillus.

In the infections produced by the four variants there was early replacement of the clumps of eosinophiles and their phagocytized bacilli by large mononuclear cells (Fig. 10). This was reflected in the peripheral blood cell count by an increase in monocytes which rarely reached its peak before the 42nd hour. From then on the monocyte curve swung back and forth, crossing the lymphocyte level at irregular intervals.

After the disease had been well established, tubercle bacilli were seen at times in uninvolved parenchyma, in all probability cast off from the older tubercles and forming new foci which proceeded to carry on the same cycle of progression. The following case may add in making this point clearer.

A chicken infected a month previously with the smooth S variant, was killed at the height of a sudden sharp increase in eosinophiles. Sections of the liver displayed scattered areas of very early tubercle formation, consisting of clumps of eosinophiles, some of which had been replaced in part by large mononuclear cells. In other portions of the parenchyma were older tubercles which had undoubtedly given origin to this particular spread of the disease. In this case the eosinophiles of the peripheral blood stream did not indicate beginning abscess formation of pre-existing tubercles but an early stage of new tubercle formation.

It seems that there was no considerable difference in tubercle formation between the four types of infection until the establishment of a definite lesion composed of large mononuclear cells. Then certain characteristics would be assumed which were especially striking in the lesions set up by the S and F.S. variants, whereas the relatively benign chromogenic Ch and R tubercles were similar and showed little evidence of progression.

The differences between the smooth S and flat smooth F.S. tubercles were most striking during the 3rd, 4th and 5th weeks of the disease. It was then that the acute, rapidly progressing exudative nature of the smooth S lesion became apparent and active proliferation of the mononuclear type cells occurred in response to the bacilli. The result was a lesion which rapidly invaded the parenchyma in all directions, assuming an irregular outline as it did so (Fig. 13). These tubercles were most striking in the liver where their margins were usually sharp, well demarcated and, with the exception of rare intervening lymphocytic and plasma cells, lying immediately adjacent to a layer of hepatic cells undergoing rapid dissolution. Staining revealed only a few bacilli present in the lesion, either in rods or branching forms (Fig. 25). The flat smooth F.S. tubercles, in contrast, gave the appearance of

a true foreign body reaction, although there was of course a certain "toxin" response as shown by the formation of the hyalin substance resembling fibrin, which was of the nature of reticulin (Fig. 14). In this lesion the cellular reaction was probably due to the presence of rapidly multiplying bacilli which resisted digestion. On media of various kinds these F.S. variants grew approximately six times as fast as did the S bacilli. We are inclined to believe that their reproduction *in vivo* continued at the same rapid rate as that *in vitro*. Comparing these bacteria with the smooth S variant, organism for organism, they probably produce much less "toxic" material of less potency. It seems that F.S. variants, by their rapid rate of multiplication and by their greater ability to resist digestion by cellular enzymes, due to higher lipin content, soon flourish in great numbers within the mononuclear phagocytes. Acid-fast stains at this stage reveal them in numerous, large, closely compacted clumps, often showing a radial arrangement of the individual bacillary rods (Fig. 26). Consequently, though the "toxin" output per bacillus may be lower than in the case of the smooth S organisms yet by their rapid multiplication and increase of numbers they bring about a progression of the disease. Conversely, the smooth S bacillus possesses a lower lipin content. These individual organisms are probably more susceptible to the action of the digestive cellular enzymes of the mononuclear phagocytes. This leads to their destruction, setting forth free "toxin" which gives to the tubercle formation its acute and malignant characteristics.

In the 3rd week the tubercles in the S-infected chickens revealed the beginning of abscess formation by an influx of eosinophiles wandering towards the center of the lesion. In the F.S. chickens this occurred much later (4th to 5th weeks). Shortly thereafter a small area of necrosis appeared in the central portion of both types of tubercles which gradually increased in size.

The production of reticulin in the S and F.S. type tubercle revealed further evidence of differences in the underlying factors governing the pathogenesis of these lesions. The former, the S tubercle, showed very little increase or even a decrease of reticulum structure lying in relation to the lesion (Figs. 17 and 11), whereas in the latter, the F.S. tubercle, there was usually an increased reticulum formation which was quite marked about the periphery (Figs. 18 and 12).

In those fowls which happened to survive 6 weeks, there was usually marked abscess formation with caseation, necrosis and formation of daughter tubercles leading to conglomerate tubercle formation, and spreads throughout the uninvolved tissue. After the tubercles had become so far advanced, which ordinarily was the exception with S and F.S. infection, the two diseases were hardly distinguishable by their microscopic appearance (Figs. 21 and 22).

The pathogenesis of the R variant differs greatly from the above two types.

From the observations made it appeared as though this organism at times possessed a low grade of virulence manifested by very limited tubercle formation of comparatively benign nature (Fig. 16). At other times it was completely innocu-

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prominent around a tubercle, silver stains would demonstrate abundant reticulum, the fibers forming a closely compacted network about the lesion. In many instances the fibrils of this network corresponded to the finer bands of the hyalin material appearing in the phloxin-methylene blue stain. In other sections it could be definitely seen that parts of the hyalin material remained unaffected by silver impregnation, leaving open spaces between the reticulum fibrils. This raised the point whether this homogeneous substance remained unimpregnated only because it was a precursor of reticulum proper and its argyrophilic nature would only become apparent after certain changes had taken place, perhaps merely a simple process of condensation and separation into smaller fibrillar divisions.

It could be definitely seen that the deposit of reticulin was much greater about the flat smooth F.S. tubercles, marking them off and circumscribing them very definitely from the surrounding parenchyma (Figs. 18 and 12). In the S tubercle, no such formation was present, in the silver preparations it was at times difficult to tell just where the tubercle margins began (Figs. 17 and 11). Often the normal reticulum about these S lesions appeared broken up into separate lengths with occasional granular formation indicating an active destructive effect. The reason for and the mechanism of this fragmentation of reticulum in this type of tubercle is not definitely understood. It is suggested, as already stated, that it is due to either a specific "toxin" liberated from the bacillus or to the proteolytic enzymes of the eosinophiles which play such a prominent part in the S infection. In making the above comparisons we used tubercles from the same tissues, stained together in the same dye container. These differences were best studied in the liver, bone marrow and lung. In the spleen, postmortem autolysis interfered quite easily with reticulum preservation. We found it always necessary to fix tissues immediately after death for the best results. Animals found dead usually showed too much postmortem degeneration of the reticulum to be of any value.

In the rough R and chromogenic Ch tubercles, silver impregnation revealed very little evidence of either the formation of a new or destruction of the normal reticulum. The pathological process was evidently of too benign and limited a character to seriously affect this structure (Figs. 20 and 19).

It would appear that in the pathogenesis of the flat smooth F.S. infection, there is a rapid reticulin production that distinguishes this type of tubercle formation from the acute exudative smooth S type.

In the latter the reticulum about the periphery was never increased in amount and often showed signs of disintegration. It seemed as though one of the important defense mechanisms against invasion by the S variant, the reaction of the reticulo-collagenous apparatus, was definitely suppressed or destroyed. In the flat smooth F.S. type this production of reticulum about the periphery of the lesion occurred at an early stage of the disease. Accepting Mallory and Parker's (7) observations that reticulum is collagen, occurring as separated fibrils or delicate strands which are always a continuation, one from the other, we must interpret this as the orderly

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physical properties, their control over tubercle formation and the ability of the F.S. variant to resist destruction and produce the foreign body type of lesion. The two variants were cultivated on the same batch of Proskauer and Beck's fluid media and approximately the same amount of inoculum introduced into each flask. The cultures were incubated for 10 weeks and the bacilli harvested by filtering through filter paper, collecting the solid masses of organisms and drying them *in vacuo* over sulfuric acid until a constant weight was obtained.

The original dry weight from the thirteen bottles of F.S. organisms was 6.3110 gm. and that from twenty-four bottles of S bacilli, 2.0814 gm. The lipin yield from the former was 1.271 gm. or 20.14 per cent and from the latter 0.3128 gm. or 15.03 per cent.

We did not attempt an accurate or detailed analysis of the chemical properties of the bacillus but merely an approximate idea of the differences in the lipin fraction of the two variants. More detailed study of the chemical properties, including the protein and carbohydrate constituents, are being contemplated.

SUMMARY

In the preceding pages we have presented evidence which we believe furnishes new light on the disease process in avian tuberculosis. From a well known strain of avian tubercle bacillus, A₁, four variants have been dissociated, each manifesting distinct colony topography and physical and chemical characteristics. From these studies we have learned that the variants are sometimes unstable, not only *in vitro* but also *in vivo*; and that this characteristic is one of the prominent factors influencing both the advancement and retrogression of the disease.

The four variants remain fairly stable *in vitro* provided they are cultivated on proper culture media. About 80 per cent smooth S and flat smooth F.S. colonies will develop true to type on egg media; and rough R and the chromogenic Ch when cultivated on glycerine agar media, in about the same percentage.

An early non-specific eosinophilia followed inoculation, no matter which variant was used. This usually subsided by the 18th hour.

The early stages of tubercle formation produced by all the variants appeared similar. First there appeared an aggregation of eosinophiles and their ingested bacilli within the tissues and then followed replacement by large mononuclear cells which wandered in and phagocyted both eosinophiles and bacilli. After the formation of tubercles com-

- Monocytes, morphological appearance, effect of proteolytic digestion products, 689
 —, multiplication, effect of proteolytic digestion products, 689
 Morphology, *Bacterium shigae* cultivated on media favorable to development of filtrability and life cycle forms, 165
 —, monocytes, effect of proteolytic digestion products, 689
- N**ERVOUS system, central, localizations of virus of poliomyelitis in, during preparalytic period, after intranasal instillation, 933
 — — in vitamin deficiency, lesions, 215
 Nitrogen metabolism, effects of anterior pituitary extracts, 349
 Nodules, subcutaneous, in rheumatic fever and rheumatoid arthritis, 845
- Nutritional encephalomalacia in chicks, influence of age upon susceptibility, 365
 — — — —, influence of breed upon susceptibility, 365
 — — — —, influence of growth upon susceptibility, 365
- O**PSONIC action of normal and immune sera based on experiments with pneumococcus, 527
- Oxycephaly and allied conditions in man and in rabbit, 967
- P**ARALYSIS, preparalytic period, localizations of virus of poliomyelitis, after intranasal instillation, in central nervous system during, 933
 Pathogenicity, pseudorabies virus, modified by animal passage, 925
- Pathology, tuberculosis, avian, with special reference to disease produced by dissociated variants, 239
- Photomicrographic analysis, motion, of mechanism of lysis of *Bacillus megatherium*, 279
 — — — —, mechanism of lysis of *Bacterium coli*, 279
- Pigment, bile, overproduction, resulting from splenectomy in bile fistula dogs, 399
- Pituitary body, anterior lobe, extracts, effects on nitrogen metabolism, water balance, and energy metabolism, 349
 — —. *See also* Prolan.
- Plants, antigoutous substances in, 121
- Pneumococci, strains other than Types I, II, and III, immunity reactions of human subjects, 95
- Pneumococcus extracts, use in effecting transformation of type *in vitro*, 265
 — infection, dermal, therapeutic mechanism of antipneumococcic serum, 139
 —, opsonic action of normal and immune sera, 527
 — pneumonia, Type I, value of skin test with type-specific capsular polysaccharide in serum treatment, 617
 —, tropic action of normal and immune sera, 527
 — Type I, polysaccharide, active immunization, 21
 — — II, polysaccharide, active immunization, 21
 — — III, polysaccharide, active immunization, 21
- Pneumonia, lobar, allergic, 111
 —, Pneumococcus Type I, value of skin test with type-specific capsular polysaccharide in serum treatment, 617

increase in numbers of bacilli, terminating with death usually during the 5th and 6th weeks. The manifestations of "toxin" damage shown in S disease were not so marked until the approach of the end-stage. We interpret the type of lesion produced by the F.S. variant as a foreign body tubercle to distinguish it from the acute "toxic" nature of the S. A quantitative chemical determination showed that this organism contained 20.14 per cent lipin whereas in the S variant it was only 15.03 per cent.

Following inoculation with R variant the initial eosinophilia returned to normal within 24 hours. There was then an increase of both lymphocytes and monocytes, the latter exceeding the former for a time. Later, usually after the 2nd month, the lymphocytes again became predominant over the monocytes, approaching the normal base line. Eosinophiles failed to react. This blood picture indicated a successful resistance against the infecting organisms, and was supported by the pathological findings. None of the chickens of this group died during the period of study (79 days) although infecting doses of 0.25 mg. were used. Necropsies on four chickens revealed no macroscopic evidence of disease approximately 11 weeks after inoculation. Microscopic lesions were present in the spleen and liver of some cases. In chickens inoculated 11 weeks previously these tubercles consisted only of very small, discrete clumps of degenerating mononuclear cells often surrounded by a border of lymphocytes. Necrosis, abscess formation or caseation was not found. Their small size and appearance was evidence of their retrogressive character. Isolated giant cells were rarely seen. It was impossible to find bacilli within these tubercles. In one instance it was possible to show complete innocuousness of this organism in the tissues, as far as producing recognizable tuberculous lesions. Reticulum played little part in the development of the lesion and it was neither increased or destroyed.

Following inoculation with the chromogenic Ch variant the initial eosinophilia declined by the 24th or 42nd hour count and from then on remained low. The lymphocytes showed little decrease. A prominent monocytosis of prolonged character usually occurred during the 3rd to 6th weeks and was then displaced by a return of the lymphocytes. By the end of the period of observation the differential count had closely approached the normal values. Again the blood picture

- Poliomyelitis, immunological relation to louping ill, 955
 — virus, localizations in central nervous system during preparalytic period, after intranasal instillation, 933
 Polysaccharide, *Pneumococcus* Type I, active immunization, 21
 —, — — II, active immunization, 21
 —, — — III, active immunization, 21
 —, type-specific capsular, value of skin test in serum treatment of Type I pneumococcus pneumonia, 617
 Precipitin reaction, 373
 Prolan, hypophyseal substance combined with, to give increased gonadotropic effects, 897
 Protein fractions of antipneumococcal serum, and unrefined antipneumococcal serum, therapeutic effects compared, 139
 — intake, effect of restriction on serum protein concentration, 705
 —, serum, concentration, effect of restriction of protein intake, 705
 Proteins, manner of removal from normal joints, 419
 Proteolytic digestion products, effect on morphological appearance of monocytes, 689
 — — —, on multiplication of monocytes, 689
 Pseudorabies virus, pathogenicity modified by animal passage, 925
 Pylorospasm and gastric retention experimentally produced in rats, 597
 Pyogenic bacteria, inflammation a factor in mechanism of invasiveness, 977
 REACTION, precipitin, 373
 Renal. See Kidney.
 Resistance to infection, acquired factors, 793, 819
 — — —, development of resistant and susceptible lines of mice through selective breeding, 793
 — — —, inherited factors, 793, 819
 Resistant and susceptible mice, *Bacillus enteritidis* infection, comparison with respect to fertility, weight, and susceptibility to various routes and types of infection, 819
 — — —, development through selective breeding, 793
 Rheumatic fever and rheumatoid arthritis, comparative study of subcutaneous nodules, 845
 Rheumatoid arthritis and rheumatic fever, comparative study of subcutaneous nodules, 845
 Rickettsia, killed, active immunization against typhus fever, 381
 —, — formalinized, properties of serum of horse immunized with, 391
 Roentgen rays. See X-ray.
 SALTS, inorganic, enhanced lethal effects of x-rays on *Bacillus coli* in presence of, 335
 Secretion, renal, cellular mechanisms, functional phase, studied by extravital method, 459
 —, — —, structural phase, studied by extravital method, 435
 —, — — —, studied by extravital method, 435, 459
 Sera, immune and normal, analysis of opsonic action based on experiments with pneumococcus, 527
 —, — — —, analysis of tropic action based on experiments with pneumococcus, 527
 —, monovalent diagnostic, prepared from fresh and stock strains of meningococcus, 561

here may bring about the proper clinical interpretation of the course of human tuberculosis.

CONCLUSIONS

1. Four variants, S, F.S., R and Ch dissociated from an avian tubercle bacillus, A₁, have been described.
2. They have different physical and chemical properties.
3. The leucocytic response in S and F.S. is of acute type while that produced by R and Ch variants is indicative of a chronic, healing tuberculosis.
4. The tubercle formed by S is of an acute, "toxic" type, the F.S. more of a foreign body type and that of R and Ch, relatively benign.
5. The S variant is by far the most virulent and is closely followed by the F.S. type. The R and Ch variants are comparatively avirulent.

We wish to acknowledge our appreciation to Drs. E. R. Baldwin and Lawrason Brown for their interest and helpful suggestions.

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EXPLANATION OF PLATES

PLATE 19

Variants of the avian tubercle bacillus cultivated on gentian violet egg media.

FIG. 1. Smooth S colonies, 30 days' growth. Actual size.

FIG. 2. Chromogenic Ch colonies, 30 days' growth. Actual size.

FIG. 3. Smooth S and chromogenic Ch colonies, 90 days' growth. Actual size.

FIG. 4. The two variants, (a) S and (b) F.S., 60 days' growth. $\times 2$.

FIG. 5. Flat smooth F.S. colonies, 60 days' growth. Actual size. Note the spreading appearance.

FIG. 6. Rough R colonies, 45 days' growth. Actual size.

FIG. 7. Rough R colonies, 60 days' growth. $\times 2$.

FIG. 8. Rough R colonies, 90 days' growth. Actual size.

PLATE 20

FIG. 9. Liver of a chicken inoculated with S variant. Early tubercle, not more than 18 hours old, consisting of simple aggregation of eosinophiles containing phagocytized bacilli. $\times 695$.

- Monocytes, morphological appearance, effect of proteolytic digestion products, 689
 —, multiplication, effect of proteolytic digestion products, 689
 Morphology, *Bacterium shigae* cultivated on media favorable to development of filtrability and life cycle forms, 165
 —, monocytes, effect of proteolytic digestion products, 689
- N**ERVOUS system, central, localizations of virus of poliomyelitis in, during preparalytic period, after intranasal instillation, 933
 — — in vitamin deficiency, lesions, 215
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 — — —, influence of growth upon susceptibility, 365
- O**PSONIC action of normal and immune sera based on experiments with pneumococcus, 527
 Oxycephaly and allied conditions in man and in rabbit, 967
- P**ARALYSIS, preparalytic period, localizations of virus of poliomyelitis, after intranasal instillation, in central nervous system during, 933
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 Pigment, bile, overproduction, resulting from splenectomy in bile fistula dogs, 399
 Pituitary body, anterior lobe, extracts, effects on nitrogen metabolism, water balance, and energy metabolism, 349
 — —. See also Prolan.
 Plants, antigoitrous substances in, 121
 Pneumococci, strains other than Types I, II, and III, immunity reactions of human subjects, 95
 Pneumococcus extracts, use in effecting transformation of type *in vitro*, 265
 — infection, dermal, therapeutic mechanism of antipneumococcic serum, 139
 —, opsonic action of normal and immune sera, 527
 — pneumonia, Type I, value of skin test with type-specific capsular polysaccharide in serum treatment, 617
 —, tropic action of normal and immune sera, 527
 — Type I, polysaccharide, active immunization, 21
 — — II, polysaccharide, active immunization, 21
 — — III, polysaccharide, active immunization, 21
 Pneumonia, lobar, allergic, 111
 —, Pneumococcus Type I, value of skin test with type-specific capsular polysaccharide in serum treatment, 617

Note little change in reticulum structure about the lesion and resemblance to Ch tubercle in Fig. 19. $\times 390$.

Silver impregnation of above four sections according to Foot and Foot.

PLATE 23

FIG. 21. Spleen of chicken inoculated with F. S. variant dying on the 69th day. Note large central area of caseation bordered by a zone of giant cells and degenerated large mononuclear cells. Only a few large remnants of the peripheral, fibrillar, hyalin material remain. $\times 53$.

FIG. 22. Spleen of a chicken inoculated with S variant and killed after 54 days. The most advanced type of tubercle seen. There is extensive caseation of the central portion marked off by a zone of giant cells and degenerated large mononuclear cells. Note resemblance of the two end-stages in Figs. 21 and 22 to each other. $\times 53$.

FIG. 23. Spleen of a chicken inoculated with R variant and killed after 78 days. This is an end-stage so far as our period of observation was concerned. The small clump of large mononuclear cells comprising this lesion typifies successful resistance on the part of the host. Note absence of necrosis or any tendency to abscess formation, also the marked limitation in size. $\times 390$.

FIG. 24. Spleen of a chicken inoculated with Ch variant and killed after 78 days, also the end-stage. Note lack of progression towards abscess formation, limited size and the close resemblance to the R tubercle of the same age in Fig. 23. $\times 390$.

The above four sections were stained with phloxin-methylene blue.

PLATE 24

FIG. 25. Spleen of chicken inoculated with S variant, showing a few bacilli in rod forms within a tubercle. $\times 1,000$. Carbofuchsin, phloxin-methylene blue stain.

FIG. 26. Spleen of chicken inoculated with F.S. variant showing many organisms arrayed in clumps throughout the tubercle. The magnification is lower than in Fig. 25 so that the entire tubercle can be included. $\times 390$. Gram-Weigert stain.

FIG. 27. Spleen of chicken inoculated with S variant, showing a tubercle containing only branching forms. $\times 865$. Carbofuchsin, phloxin-methylene blue stain.

FIG. 28. Ch variant after 2 weeks' cultivation on glycerine agar, but when grown on glycerine gentian violet egg media the morphology was as in Fig. 29. $\times 865$. Carbofuchsin.

FIG. 29. S variant cultivated on gentian violet egg media for 3 weeks. $\times 865$. Carbofuchsin.

FIG. 30. Direct smear from spleen of chicken inoculated with S variant and killed after 37 days. Note granules within the branching organism. $\times 1,300$. Weiss stain.

FIG. 31. Direct smear of spleen from same chicken. Note prominent granules in one branching form and lack of granules in other. $\times 865$. Carbofuchsin, brilliant green stain.

ous within the animal body so far as producing any microscopic tubercles. In chickens, infected with R variant, when we found the types of tubercles previously described, they were always of comparatively minute size and in comparison with the tubercles produced by the virulent S and F.S. variants were apparently stationary as far as progression goes. They were tubercles, first, because of their appearance; second, because of their location, and third, because the causative agent could be transmitted from one chicken to another.

The reticulum about these tubercles did not appear to enter much into the reaction (Fig. 20). It appeared to be no different from the reticulum structure elsewhere in the parenchyma, and was neither increased in amount, thickened to form collagen nor destroyed. It had apparently received insufficient stimulation to become active and participate in the slight amount of tissue reaction that had occurred.

The chromogenic Ch variant infection resembles the R variant very closely in its pathogenic properties.

Its virulence was of very low grade and remained almost constant. At no time in our experiments during the period of observation (up to 3 months), were we able to kill with this bacillus using doses up to 0.5 mg. The lesions, when any were produced by them, were similar to those produced by the R bacillus. 11 and 12 weeks after inoculation the Ch tubercles were no more advanced than the S or F.S. tubercles of 1 week's duration (Fig. 15). It was also questionable, as with the R tubercles, whether these tubercles would progress to caseation for the reason that there was insufficient bacillary influence to attract eosinophiles which would lead to abscess formation. The reticulum about these lesions remained quite inactive and in every way similar to that described in the R tubercle formation (Fig. 19).

Reaction of the Reticulum.—Our attention was centered on the striking tubercle formation set up by the flat smooth F.S. variant and an attempt was made to identify the nature of the peripheral hyalin-like zone surrounding the tubercle (Fig. 14). Because of the peripheral arrangement and the curly, waving nature of the strands often seen comprising it, we were led to believe that this substance was related to collagen. The other possibilities were fibrin or the products of lysed cells. It did not resemble fibrin which generally lies in coarse, straight fibrils with nodal thickenings usually located in the center of the tubercle. The clear translucent hyalin appearance was against its being the product of lysed cells; ordinarily one would also expect to find in such material the fragments and remains of broken down cell structures, especially the nuclei. After staining by Gram-Weigert's method this material assumed a red or azur-red tint, whereas fibrin should ordinarily stain blue. With Mallory's aniline blue collagen fuchsin stained it red. This was good evidence for the collagenous nature of the substance and silver impregnation by the method of Foot and Foot apparently proved the point because fibrin, according to prevalent opinion, is never impregnated by silver. Whenever this hyalin peripheral zone was

initiation of a mechanism of defense which attempted to limit the spread of the lesion. In the smooth S infection the "toxic" action inhibited this step by directly depressing the reticulon-secreting activity of certain cells and the proteolytic enzymes of the polymorphonuclear leucocytes by actively destroying reticulum already preformed. In the flat smooth F.S., or a foreign body tubercle, there was a chance for this process to get temporarily under way in an attempt to wall off the lesion with a zone of collagen and prevent its spread. Unfortunately, the rapid bacillary growth from within became powerful enough to overcome this temporary check. The manifestations described were present only during the early part of tubercle formation. After about the 6th week, when necrosis and extensive abscess formation had occurred (Figs. 21 and 22), there was little to differentiate the two forms histologically except the occasional persistence of portions of the reticulum pattern about the periphery of large F.S. tubercles in the liver, bone marrow and the lungs. In most cases, however, this formation began to break up and disappear as the necrosis advanced until it was either no longer visible or only small remnants were left.

Morphological Variations of the Organism

The occurrence of the branching form of the avian tubercle bacillus was known soon after its discovery. In our studies microorganisms of such morphology were observed only in chickens infected with smooth S variant.

Two chickens were inoculated with a suspension of S variant consisting of slender rods (Fig. 29). One was killed on the 37th day and only branching forms were found in the tubercles (Fig. 27). The other, killed on the 43rd day, demonstrated no bacilli of any form in the lesions, yet their presence was proved by an abundant cultural growth which a subsequent inoculation proved to be of high virulence.

The occurrence of the branching form in no way altered the progression of the disease; it remained rapid and the tubercle formation was of the characteristic acute exudative S type. There was likewise no departure from the conventional septic blood picture so characteristic of this group. At present we are inclined to believe that the branching state was apparently only a temporary and variable phase in the life cycle of the organism. A study dealing particularly with this question is at present in progress.

Analysis of Lipin Content of S and F.S. Variants

It has been pointed out that the dissociated variants S and F.S. produced different pathological pictures in which it seemed that the microorganisms played a greater part than the host.

The chemical analysis was limited to a determination of the lipin content of the two variants to see if differences therein would not help to explain the variation in

posed only of large mononuclear cells, certain differences between the virulent and avirulent variants became apparent.

There was also a direct relationship between the dosage and the extent of the disease. In our experience from 0.16 to 0.25 mg. gave the most uniform results.

If S variant was used, the early non-specific eosinophilia was followed by a second rise which continued to ascend, running parallel with the total leucocyte count. There was a slow increase of monocytes and a corresponding decrease of lymphocytes. The microscopic lesions, 2 weeks after inoculation, were composed of irregularly shaped clumps of necrosing large mononuclear cells. The margins were clear-cut and bordered by few or no lymphocytic cells. By the 3rd and 4th weeks eosinophiles began to migrate into the centers of the tubercles and abscess formation became evident. Death usually occurred after 5 or 6 weeks, although this varied with the dosage. The appearance of the lesions suggested an acute "toxic" nature, as manifested (1) by marked enlargement of the spleen and liver, (2) by the short fatal course of the disease, which never became very extensive, (3) by the presence of few organisms within the tubercles, (4) by destruction of reticulum and (5) by the marked response of the blood leucocytes.

After inoculation with F.S. variant the early non-specific eosinophilia disappeared, returning again at the terminal stage, 5 to 6 weeks later. The number of eosinophiles then ascended sharply, accompanied by a marked leucocytosis which continued until death. During this eosinophilia a monocytosis occurred and lymphocytes fell away rapidly.

During the 2nd week, clumps of closely packed large mononuclear cells containing masses of phagocyted bacilli were seen. In the 3rd and 4th weeks there appeared a prominent peripheral zone of hyalin, collagenous-like material associated with an increased reticulum formation which apparently walled off the lesion. The bacilli within the tubercle multiplied rapidly, it seemed as though they were more resistant to destruction than the S organisms, perhaps due to their higher lipid content. As a result of their rapid increase, progression of the tubercle continued, with necrosis and abscess formation, accompanied by the dissemination of the organisms both locally and distantly to new uninvolved tissue. Wide extension of the disease followed due to the

was truly representative of the relatively benign character of the lesions. The tubercle formation, even after 93 days' duration of the disease, remained limited to small clumps of two or three large mononuclear cells showing some degeneration. Lymphocytic cells were usually present within and about the lesion. There was never any evidence of abscess formation or actual necrosis. Bacilli could not be found within these tubercles. The reticulum in relation to the lesion showed neither increase nor destruction. None of the chickens died though a dose of 0.5 mg. was used.

In three chickens infected with S variant, the blood picture did not follow the pattern set by the others similarly inoculated. Either a high monocytoysis replaced the usual eosinophilia, or there was a marked recession of the total leucocyte count followed by a return of lymphocytes towards normal percentage. The blood picture indicated the conversion of an acute process into a subacute or chronic affair. These chickens survived for 78, 79 and 110 days, respectively. An explanation for this occurred to us later when cultures recovered from them, instead of consisting of S colonies, showed the topography of the intermediate type, closely resembling the F.S. type. Evidently this was due to a reversion; *i.e.*, a loss of virulence of the organism occurring within the animal body. At necropsy the spleen and liver were somewhat enlarged and contained prominent, discrete yellow nodules resembling small shot in their size and shape, which, in one case could be lifted from the tissue by the point of the knife. Microscopically, they possessed small centers of caseation surrounded by numerous giant cells and large mononuclear cells with a thick border of lymphocytic cells. This tubercle formation could not be classified in any special group as it was too sharply circumscribed and walled off by lymphocytic cells to be considered as truly malignant in nature as the S type of lesion. Even the younger tubercles in these cases, which consisted of clumped epithelioid cells with prominent lymphocytic cell borders, could not be called acute or "toxic" in character.

At present we make only limited deductions from our observations. In the bacterial dissociation phenomenon we have at least a new line of investigation, and different variants of tubercle bacilli must be taken into consideration when planning new tuberculosis studies. With the stabilization of the human type variants, experiments such as reported

- FIG. 10. Liver of a chicken inoculated with chromogenic Ch variant. An early tubercle, not more than 36 hours old. The clump of eosinophiles is being replaced and phagocytosed by large mononuclear cells. $\times 695$.
- FIG. 11. Bone marrow (femur) of a chicken inoculated with S variant and killed on the 58th day, showing no formation of reticulum about the lesion, but scattered, broken up fibrils suggesting actual reticulum destruction. $\times 105$.
- FIG. 12. Bone marrow (femur) of a chicken inoculated with F.S. variant killed on the 33rd day. Tubercle formation with abundant peripheral reticulum can be seen.
- Figs. 11 and 12 stained by silver impregnation according to Foot and Foot.

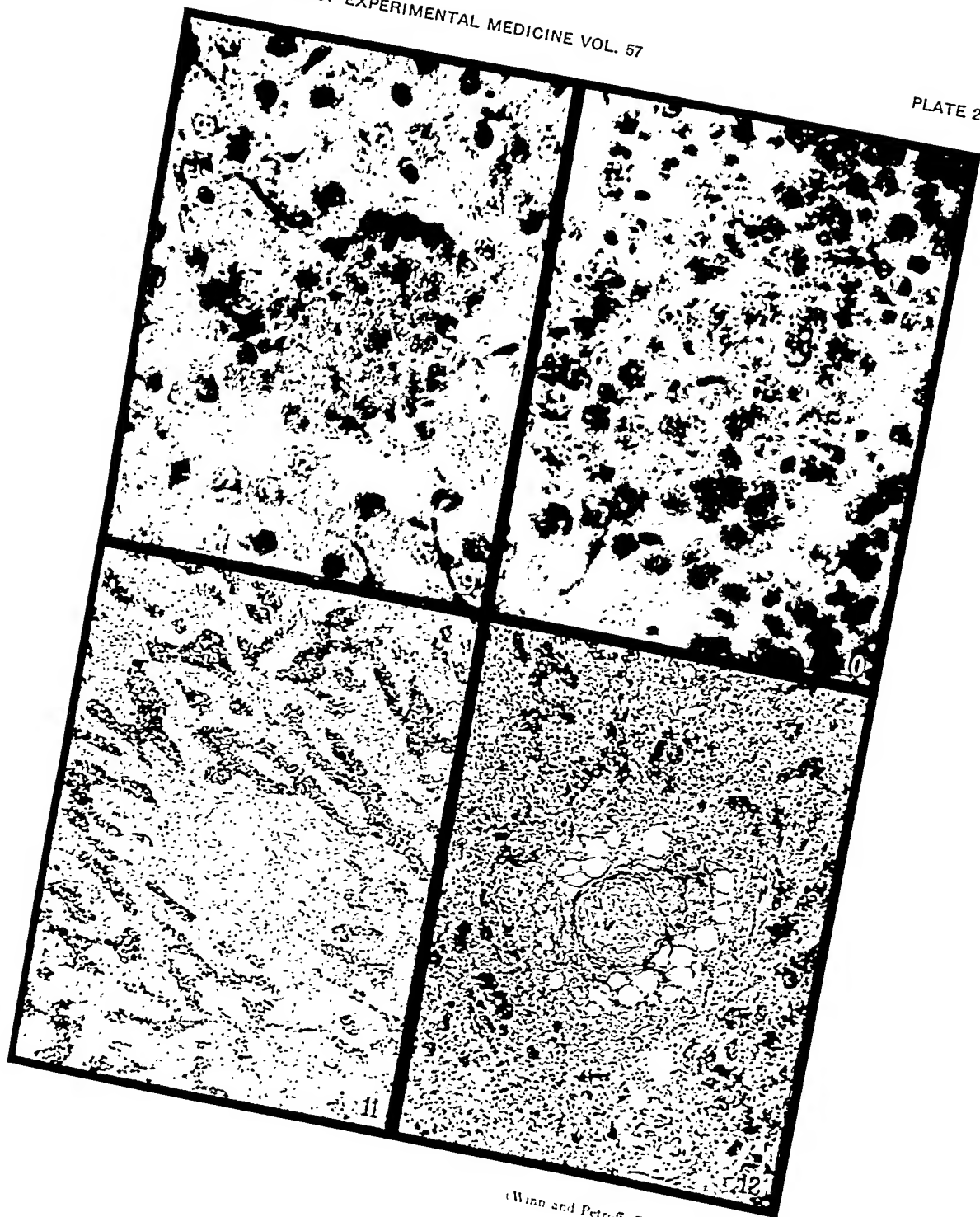
PLATE 21

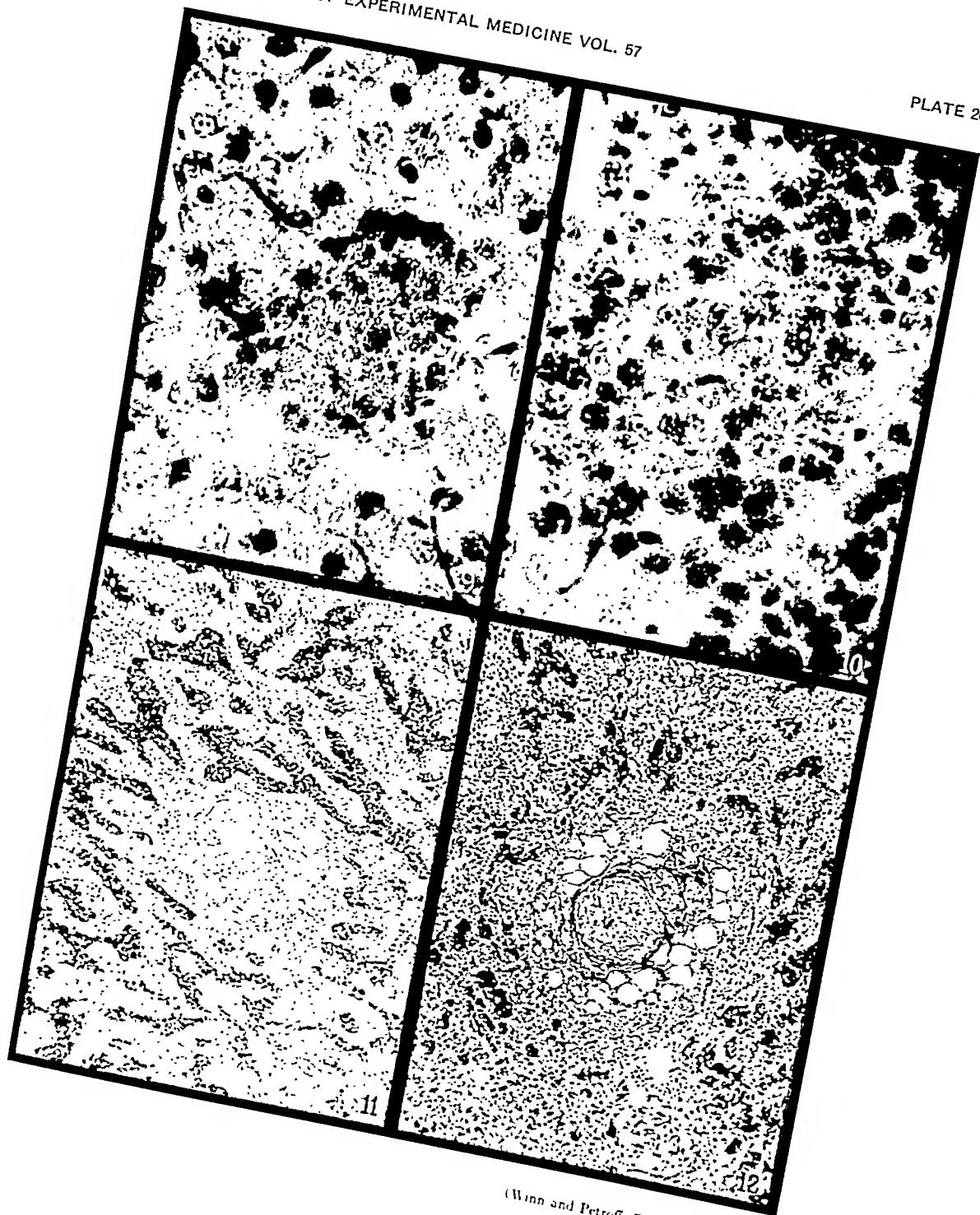
- FIG. 13. Liver of a chicken inoculated with S variant dying in 37 days. Note irregular extension of the lesion which is composed of degenerating, large mononuclear cells and a few scattered eosinophiles. The clean-cut border, absence of peripheral reaction and the dissolution and disappearance of adjacent liver cells is apparent. This is an acute, "toxic" tubercle with a malignant type of extension. $\times 390$.
- FIG. 14. Liver of a chicken inoculated with F.S. variant and killed on the 31st day. Note rounded clump of closely packed, degenerating, large mononuclear cells, with prominent peripheral zone of hyalin material and marginal fringe of lymphocytic, large mononuclear and plasma cells. This is acute, foreign body type of tubercle formation. $\times 325$.
- FIG. 15. Liver of a chicken inoculated with Ch variant and killed at 78 days. Note the discrete appearance of the lesion and small size by comparing with size of the liver cells. It consists only of a few large mononuclear cells surrounded by a fringe of lymphocytes and is of retrogressive nature. $\times 390$.
- FIG. 16. Liver of a chicken inoculated with R variant and killed at 78th day. Note the comparatively benign appearance and close resemblance to Ch type lesion in Fig. 16. It consists of a few degenerating, large mononuclear cells with little peripheral lymphocytic reaction. $\times 390$.
- The above four sections stained with phloxin-methylene blue.

PLATE 22

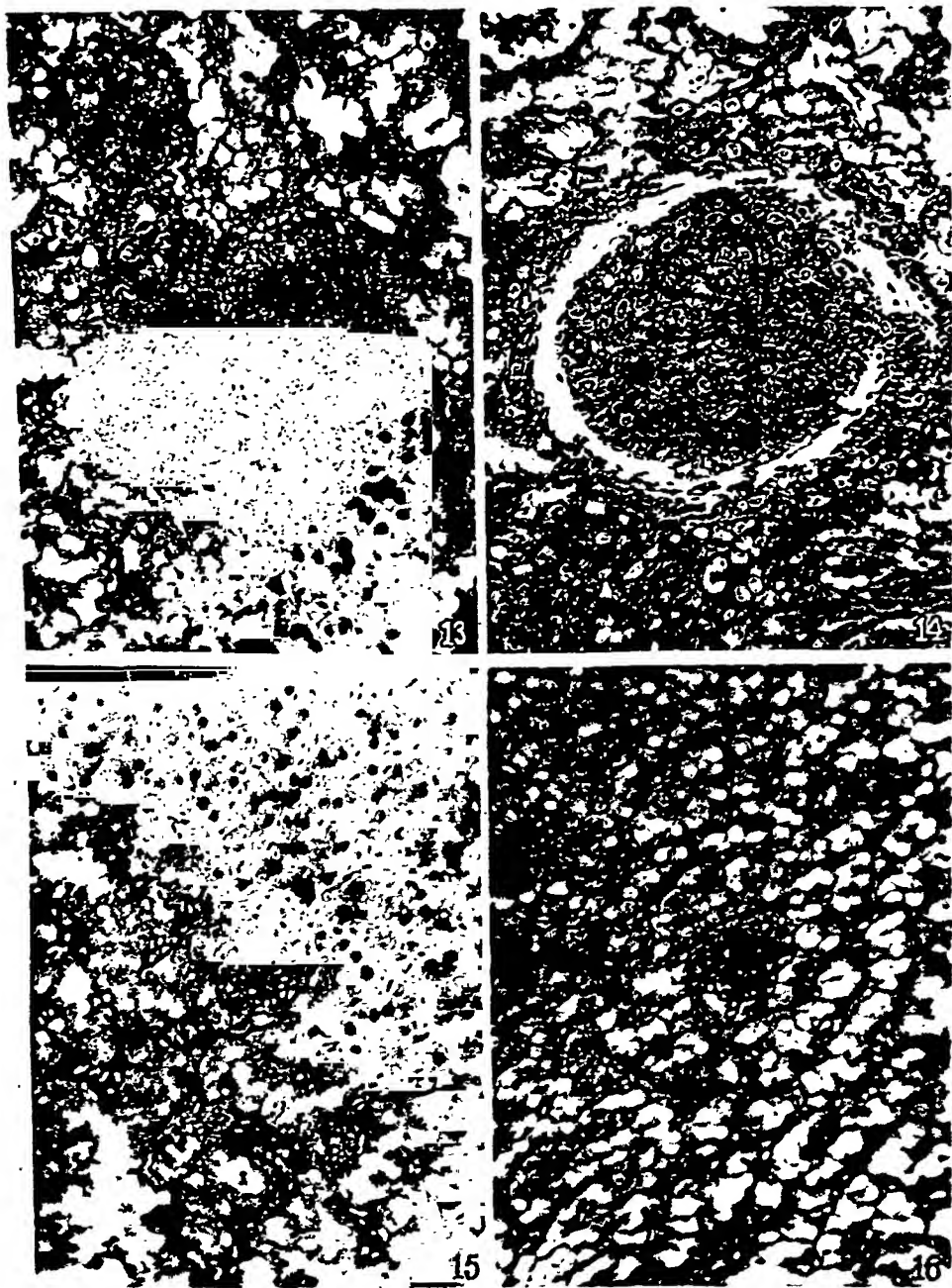
- FIG. 17. Liver of a chicken inoculated with S variant, showing reticulum about two adjoining tubercles of 19 days' duration. Note fragmented reticulum within the lesions and lack of any peripheral increase. $\times 345$.
- FIG. 18. Liver of a chicken inoculated with F.S. variant dying at 35th day, showing marked increase of reticulum about the periphery of the lesion, circumscribing it completely. $\times 260$.
- FIG. 19. Liver of chicken inoculated with Ch variant and killed at 78th day, showing neither increase nor destruction of reticulum about the tubercle. $\times 450$.
- FIG. 20. Liver of chicken inoculated with R variant and killed at 78th day.







(Winn and Petroff. Tubercle bacillus. 11)



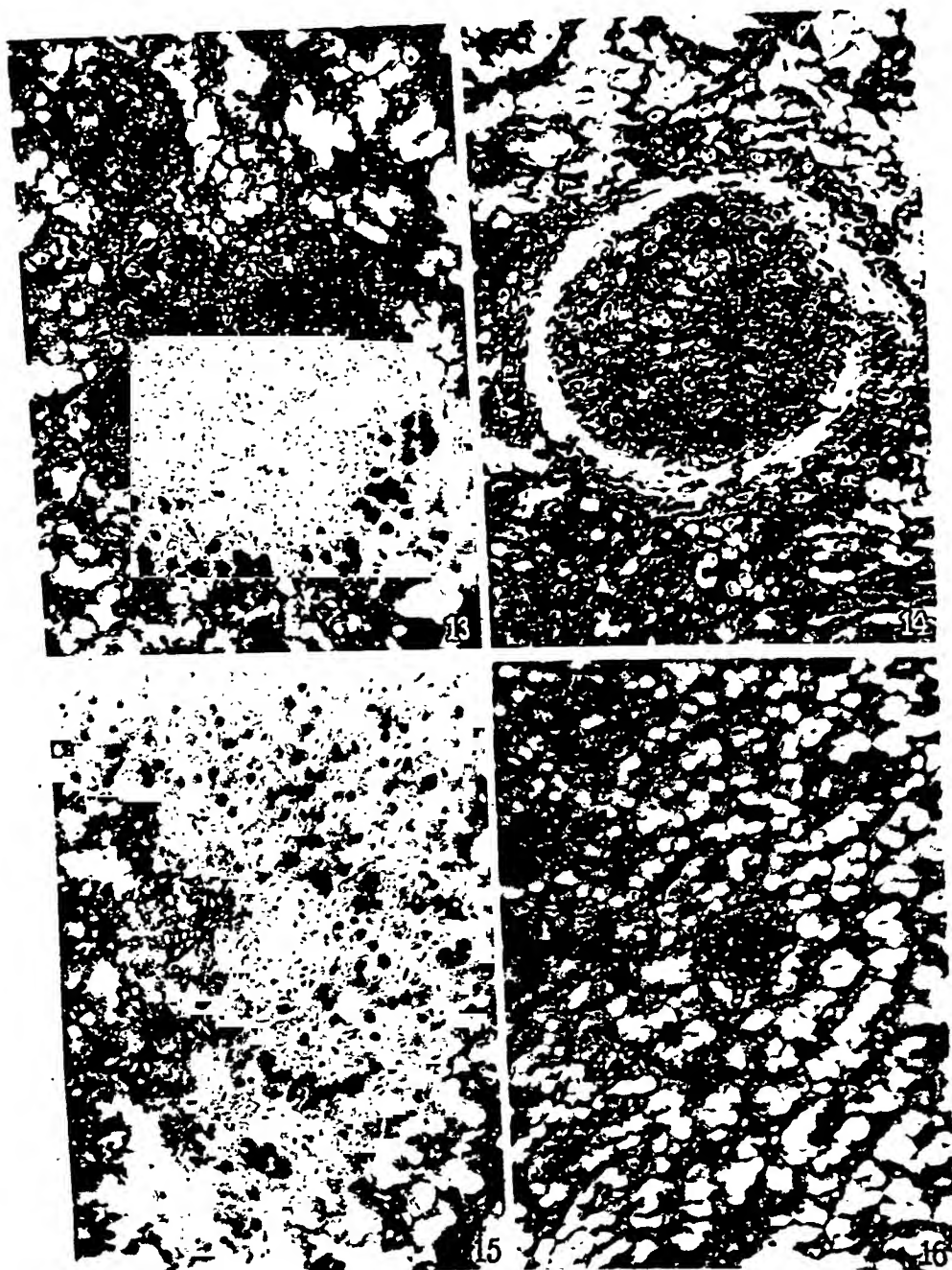
(Winn and Petroff, Tubercle bacillus, 11)

Methods

Cultures.—The cultures of R pneumococci used in the experiments were stock strains originally derived from type-specific S pneumococci by growth in broth containing 10 per cent antipneumococcus serum of the homologous type. The pneumococci from which the extracts were prepared were type-specific S strains, the virulence of which had been maintained by frequent animal passages.

Preparation of Pneumococcus Extract.—The S pneumococci used in preparing extracts were grown in meat infusion broth, pH 7.8, containing dextrose 0.01 per cent. Large inocula were used to bring about rapid and dense growth. At least 100 cc. of a moderately heavy culture was used to inoculate 5 liters of media. The cultures were grown at 37°C. for 8 to 10 hours only. The organisms from 5 liters of culture were thrown down and then taken up in 50 cc. of sterile distilled water. To this suspension was added 3.5 cc. of a sterile 10 per cent solution of sodium desoxycholate. The mixture was kept in an ice bath for 10 minutes, then brought slowly to 60°C. in a water bath. The organisms treated in this manner are quickly dissolved, forming a thick, extremely viscous gel. The preparation was kept in the water bath at 60°C. for 15 minutes to kill any surviving organisms, and to inhibit or destroy the autolytic enzymes released by dissolution of the cells. The bacterial solution was added slowly to 500 cc. of absolute alcohol previously chilled in an ice bath. A thick, stringy precipitate formed which slowly settled out on standing. The sodium desoxycholate, being soluble in alcohol, remained in the supernatant fluid. The precipitate was thrown down after 30 minutes, by centrifugation, the supernatant fluid being discarded. The sodium desoxycholate solution was thus eliminated. The precipitate was washed with alcohol to remove the last traces of the bile salt and was dried *in vacuo*, or by exposure for 12 to 14 hours to cold dry air. The dried material was then extracted in 100 cc. of sterile 0.85 per cent sodium chloride solution, made slightly alkaline by the addition of 0.5 cc. of N/10 sodium hydroxide, sealed in glass ampoules, and immersed for 15 minutes in a water bath at 60°C. The preparation was then centrifuged for 30 minutes and the insoluble residue, which contained only a small amount of the active material, was discarded. The supernatant extract was a slightly turbid, thin, opalescent fluid. The extracts were subjected to rigid tests for sterility. They were cultured in plain broth, in blood broth, on blood agar, and in media enriched by the addition of ascitic or chest fluid. In no instance were pneumococci grown from the extracts. Injected in amounts as large as 1 cc. into mice, they caused no untoward effects other than slight lethargy for a few hours. All the test animals survived.

Culture Medium.—Dawson and Sia found in their *in vitro* experiments (4, 5), that the transformation of pneumococci from one specific type to another was facilitated by the addition to the culture medium of anti-R serum. Their observations have been repeatedly confirmed



satisfactory and consistent results. Purification with charcoal was carried out as follows:

100 cc. of the extract prepared in the manner described was diluted with an equal volume of sterile 0.85 per cent solution of sodium chloride. 8 gm. of sterile powdered wood charcoal was added to the diluted extract and the mixture was shaken for 5 minutes. The preparation was then centrifuged at high speed for 30 minutes and the supernatant fluid, still containing considerable amounts of finely

TABLE I
Activity of Extract of S Pneumococci before Adsorption and Purification

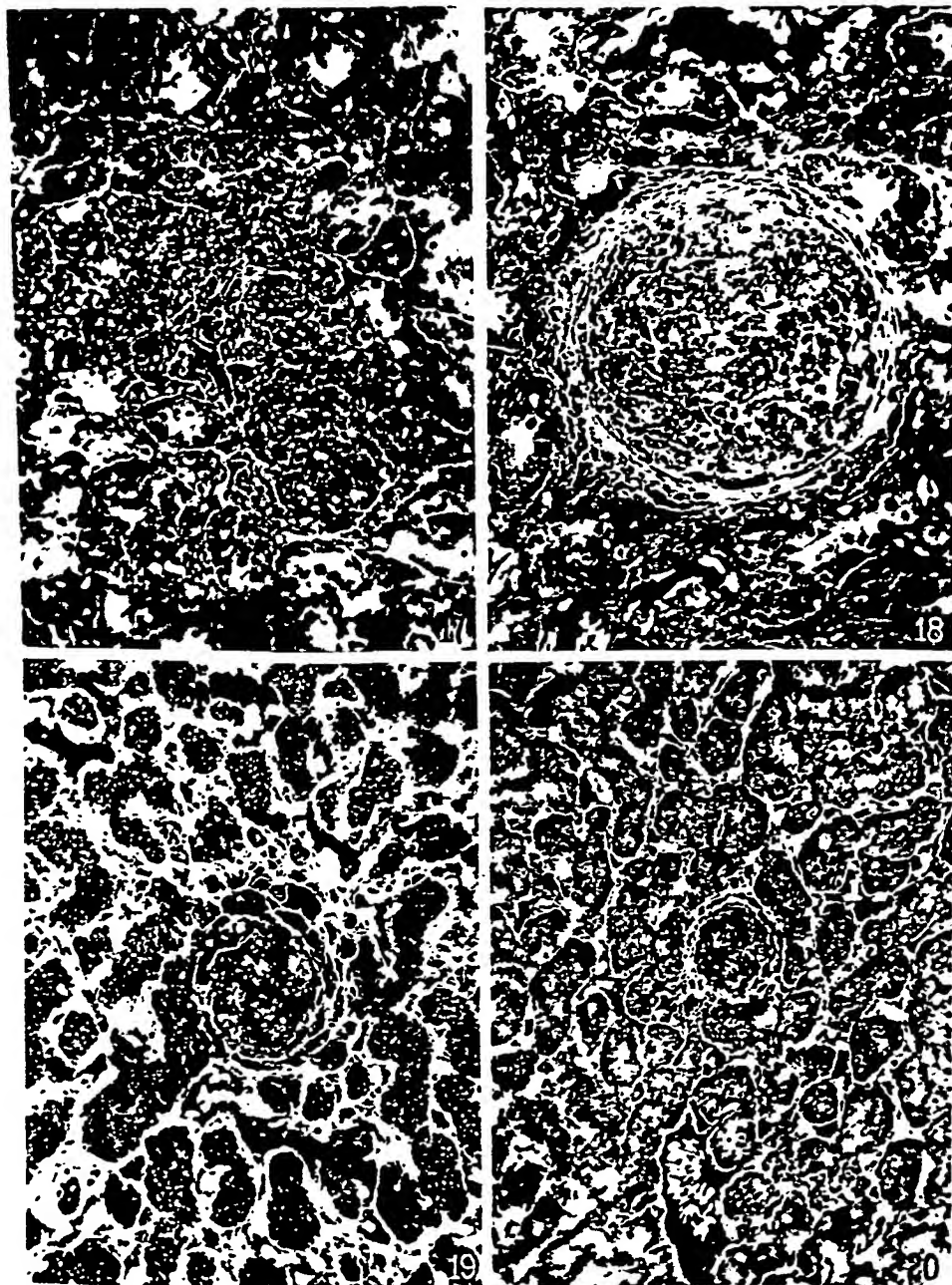
Tube	Broth	Serum factor (chest fluid)	R organism	Specific extract Type III pneumococci*	Colonies	Specific agglutination of S colonies
First culture						
1a	cc.	cc.	D-39-R†	cc.	R and S	Type III
2a	1.5	0.5	D-39-R	0.1	R only	—
3a	1.5	0.5	D-39-R	0.05	R only	—
4a	1.5	0.5	D-39-R	0.05	R only	—
5a	1.5	0.5	—	0.1	Sterile	—
First subculture						
1b	1.5	0.5	From Tube 1a	0.1	R and S	Type III
2b	1.5	0.5	From Tube 2a	0.1	R and S	Type III
3b	1.5	0.5	From Tube 3a	0.05	R and S	Type III
4b	1.5	0.5	From Tube 4a	0.05	R only	—

* Pneumococcus extract prepared by dissolving Type III S organisms in a solution of sodium desoxycholate with removal of bile salt by precipitating the extract in alcohol.

† Strain of R Pneumococcus derived from Type II S organisms.

suspended charcoal, was removed. This solution was filtered through sterile filter paper and finally through a Berkefeld V candle. The filtered extract was generally water-clear, colorless, and quite limpid.

Extracts purified by the use of charcoal were more active in effecting transformation than were the crude extracts. Moreover, it was much easier to determine when transformation had occurred in cultures containing these clear extracts. The medium was entirely clear and transparent on inoculation. Growth was granular and settled



(Winn and Petroff: Tubercle bacillus, 11)

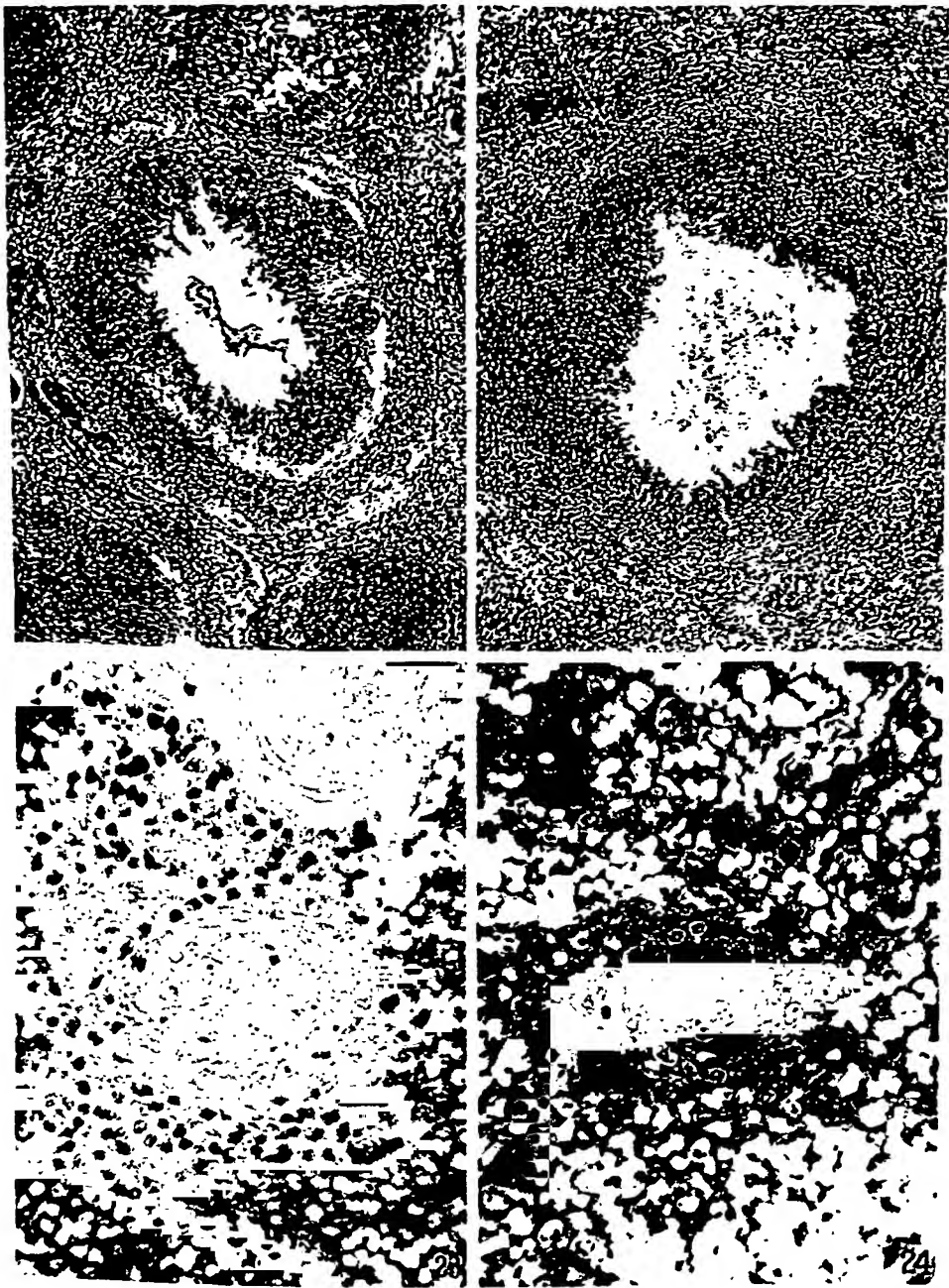
Charcoal-adsorbed extracts of Types I, II, and III S pneumococci were prepared and found to be highly active in all instances. In Table II are recorded the transformations brought about through the use of extracts after removal of considerable inactive material by charcoal adsorption.

As the results presented in Table II show, it was possible to cause R pneumococci derived from each of the three specific types to revert to their original S forms through the use of specific extracts of the homologous type. Likewise, it was possible, with one exception, to effect a selective transformation in type, whereby the R cells derived from each type of *Pneumococcus* were changed into the S forms of each of the other two specific types in the presence of the appropriate extract. It was impossible, in two attempts made, to effect transformation of R pneumococci derived from Type III S organisms into Type I pneumococci. Instead, these particular R pneumococci reverted to S forms of the original specific type from which they were derived; namely, Type III. The tendency of R pneumococci to revert to S organisms of the original type, even in the presence of a suspension of heat-killed S organisms of a heterologous type, was encountered and commented upon both by Griffith and by Dawson.

As stated in the previous paper (7), it was quite easy to convert R pneumococci derived from Type II S organisms into Type III S forms. In most instances the change occurred in the first culture within 15 to 20 hours. Other R strains changed less readily and often required two, three, and even four transfers. Thus, in the experiment shown in Table II, the R organisms derived from Type I S pneumococci were changed into Type II forms only on the third successive cultivation in the extract-containing medium.

Reprecipitation in Acetone or Alcohol.—Still further purification of the charcoal-adsorbed extracts was accomplished by precipitation in acetone.

The filtered extract, after charcoal adsorption, was precipitated by 10 volumes of acetone in the cold. The preparation, after standing 1 hour, was centrifuged at high speed for 30 minutes and the supernatant acetone discarded. The sediment was dried *in vacuo* and taken up in the original volume of sterile distilled water. Much of the precipitate remained insoluble. The insoluble residue was thrown down by centrifugation and discarded. The supernatant contained the active material without appreciable loss.



(Winn and Petroff: Tubercle leucocytes. II)

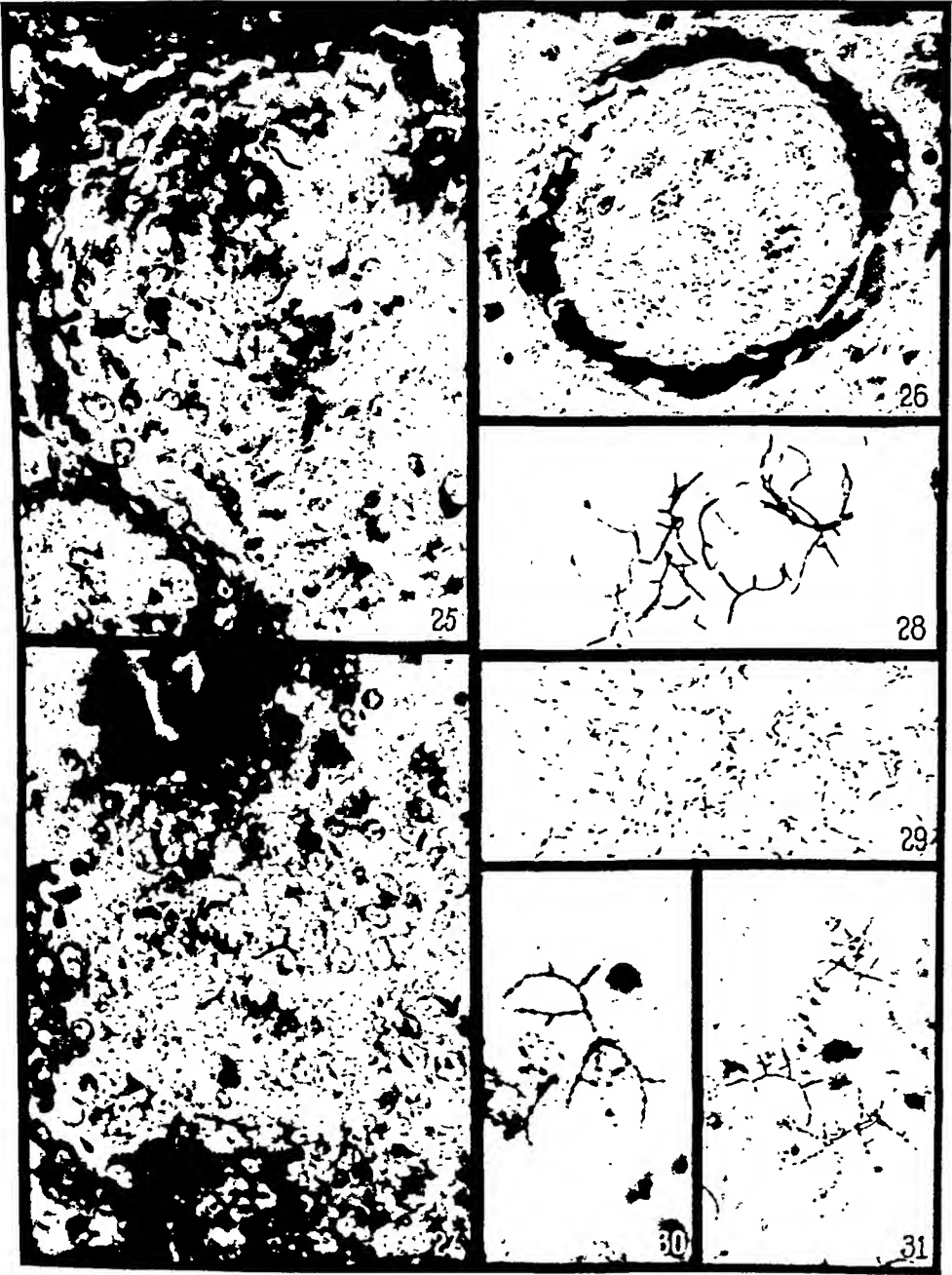
TABLE IV

Effect of Heat on the Activity of Purified Pneumococcus Extract

Tube	Broth	Serum factor (chest fluid)	R organism	Type III specific extract*		Colonies	Specific agglutination of S colonies
				Heated for 10 min. at	Amount		
First culture							
	cc.	cc.		°C.	cc.		
1a	1.5	0.5	D-39-R†	70	1.0	R and S	Type III
2a	1.5	0.5	D-39-R	70	1.0	R only	—
3a	1.5	0.5	D-39-R	70	0.5	R only	—
4a	1.5	0.5	D-39-R	80	1.0	R only	—
5a	1.5	0.5	D-39-R	80	1.0	R only	—
6a	1.5	0.5	D-39-R	80	0.5	R and S	Type III
7a	1.5	0.5	D-39-R	90	1.0	R only	—
8a	1.5	0.5	D-39-R	90	1.0	R only	—
9a	1.5	0.5	D-39-R	90	0.5	R only	—
First subculture							
1b	1.5	0.5	From Tube 1a	70	1.0	R and S	Type III
2b	1.5	0.5	From Tube 2a	70	1.0	R only	—
3b	1.5	0.5	From Tube 3a	70	0.5	R and S	Type III
4b	1.5	0.5	From Tube 4a	80	1.0	R and S	Type III
5b	1.5	0.5	From Tube 5a	80	1.0	R and S	Type III
6b	1.5	0.5	From Tube 6a	80	0.5	R and S	Type III
7b	1.5	0.5	From Tube 7a	90	1.0	R only	—
8b	1.5	0.5	From Tube 8a	90	1.0	R and S	Type III
9b	1.5	0.5	From Tube 9a	90	0.5	R only	—
Second subculture							
2c	1.5	0.5	From Tube 2b	70	1.0	R and S	Type III
7c	1.5	0.5	From Tube 7b	90	1.0	R only	—
8c	1.5	0.5	From Tube 8b	90	1.0	R and S	Type III
9c	1.5	0.5	From Tube 9b	90	0.5	R only	—
Third subculture							
7d	1.5	0.5	From Tube 7c	90	1.0	R only	—
9d	1.5	0.5	From Tube 9c	90	0.5	R only	—

* Extract of Type III S pneumococci prepared by dissolving the culture in a solution of sodium desoxycholate, precipitating in alcohol, and extracting the precipitate in saline solution.

† Strain of R Pneumococcus derived originally from Type II S organisms.



(Winn and Petroff: Tubercle bacillus. II)

through the N but also through the W type of Berkefeld filter. When the extract was acid, however, filtration resulted in complete loss of activity. The purified extracts passed through W filters were crystal-clear and colorless. They still exhibited full activity as demonstrated by the results of the filtration experiment recorded in Table V.

TABLE VI

Active Immunity Induced in Mice by Injection of Purified Pneumococcus Extract (Type I)

Mouse	Type I extract* injected†			Virulent culture of Type I pneumococci injected† May 11	Result
	Apr. 27	Apr. 30	May 4		
	cc.	cc.	cc.	cc.	
1	0.2	0.2	0.2	0.001	S
2	0.2	0.2	0.2	0.001	D 18
3	0.2	0.2	0.2	0.0001	D 18
4	0.2	0.2	0.2	0.0001	S
5	0.2	0.2	0.2	0.00001	S
6	0.2	0.2	0.2	0.00001	S
7	0.2	0.2	0.2	0.000001	S
8	0.2	0.2	0.2	0.000001	D 60

Virulence Control

Mouse	Virulent Type I culture†	Result
	cc.	
1	0.001	D 18
2	0.0001	D 18
3	0.00001	D 24
4	0.000001	D 40
5	0.0000001	D 40
6	0.00000001	D 40

* Extract prepared by dissolving Type I pneumococci in a solution of sodium desoxycholate, precipitating in alcohol, extracting the precipitate in saline solution, adsorbing with charcoal, and filtering through Berkefeld V candle.

† All injections made intraperitoneally.

S = survived; period of observation 21 days.

D = died; the numeral indicates the number of hours before death of the animal.

Soluble Specific Substance.—The purified extracts contained varying amounts of the soluble specific substance of the pneumococci from which they were prepared. However, the concentration of the type-

FURTHER OBSERVATIONS ON THE USE OF PNEUMOCOCCUS EXTRACTS IN EFFECTING TRANSFORMATION OF TYPE IN VITRO

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The fact has been conclusively proved by Griffith (1), and subsequently confirmed by Neufeld and Levinthal (2) and Dawson (3) that R pneumococci derived from S organisms of one specific type may be transformed into S forms of a different specific type. The change was first accomplished *in vivo* by Griffith, who injected into mice living R organisms derived from one type of *Pneumococcus* together with large quantities of heat-killed S pneumococci of a different type. Living S forms of the same type as that of the killed S organisms injected, were recovered from the animals.

Dawson and Sia (4, 5) succeeded later in bringing about transformation of type *in vitro*. They inoculated with minute quantities of living R organisms derived from one type of *Pneumococcus* a medium of broth containing anti-R serum and the whole heat-killed S organisms of a different type. From the cultures originally inoculated with R pneumococci, S organisms were recovered which were identical in type with that of the killed S pneumococci present in the medium. In a previous paper from this laboratory (7), experiments were described in which transformation of type had been effected *in vitro* through the use of cell-free extracts of S pneumococci. These extracts, although filtrable, were crude and contained large amounts of cellular debris now known to be unessential for the transformation. Moreover, the earlier method of preparation resulted in the loss of a considerable amount of the active substance. The present paper describes a more efficient method of extracting pneumococci, and records the results of attempts to purify further the active substance.

the soluble specific substance. Extracts which were apparently equally potent in causing transformation varied considerably in their content of soluble specific substance. It has been proved (6) that the specific capsular polysaccharide in chemically purified form, as such, is ineffective in inducing transformation in type. It seems probable therefore, that if the soluble specific substance in these extracts is concerned at all in the reaction, it is present there in a different physical state, or in combination with some other substance which confers upon it properties not found in the chemically isolated and highly purified substance.

Certain strains of R pneumococci were found to be more resistant to transformation than were others, but none were encountered which were completely refractory. Transformation of R pneumococci derived from Type II strains to the Type III S forms in the presence of Type III extract seemed to take place almost abruptly. Very rarely were transitional colonies noted. Likewise, under the influence of Type II extract, R cells derived from Type III organisms changed abruptly to Type II pneumococci. However, the change of R forms derived from Type II pneumococci to Type I S organisms was a more gradual one, and required, at times, a series of transfers in the specific extract medium. In this instance, all stages in transformation were noted in colonies plated from an individual culture. Once the change was complete, however, the newly acquired type-specific characters persisted.

The factor which is presumably common to blood serum, ascitic and chest fluid, and which is essential in the reaction, remains unknown. No experiments were successfully completed without the addition to the medium of one or another of these three related substances.

The present experiments afford additional evidence that the transformation in type is not apparent, but real, and that the changes are brought about in the presence of the extract through the specific action of a soluble constituent present in S forms of pneumococci. It is almost inconceivable that any living element in the pneumococcus cell could survive the drastic procedures employed in the preparation of the extracts. Through the action of sodium desoxycholate, pneumococci were completely dissolved so that no recognizable cellular forms remained. The extracts were heated to 60°C. for a total of 30

in this laboratory. In fact, no transformation has been possible in the author's experience without the use of serum or a serous fluid in the medium.

Hog serum and anti-R rabbit serum were used in earlier experiments. It has been found recently, however, that sterile ascitic or chest fluid is more effective than blood serum, when added to the nutrient broth in proportion of 1 to 3. The chest fluid used in the present experiments was obtained from a patient with cardiac failure and general anasarca. It was a straw-colored, clear transudate, and proved sterile on culture. Prior to use it was filtered through a Berkefeld V candle. Titrations of its anti-R properties revealed that it would agglutinate R pneumococci only in dilutions of 1-20, or, rarely, 1-40.

Cultural Technic.—The cultural technic employed in effecting the transformations was similar, save for minor changes, to that described in an earlier paper (7). Inocula, consisting of 1 drop of a 10^{-4} dilution of an 8 hour culture of R pneumococci, were added to a series of tubes containing 1.5 cc. of broth, 0.5 cc. of chest fluid, and varying quantities, 0.05 to 1.0 cc., of the specific pneumococcus extract. The cultures were grown aerobically at 37°C. for 24 hours. Transfers to fresh medium were made serially every 24 hours, 1 drop of culture being carried forward to a corresponding tube in the second series. At the time of each transfer, subcultures were made on blood agar plates for the subsequent study of the colony characteristics of the organisms. No experiment was considered negative until five serial transfers were made. Whenever smooth colonies were noted on subculture, one typical colony was transferred to blood broth and the specific type of the organisms was subsequently identified serologically.

Activity of Extracts.—Extracts made from S pneumococci by the action of sodium desoxycholate proved much more effective in inducing transformations in type than did the extracts which were formerly prepared from frozen and thawed organisms. They were effective in high dilution, and even failed to induce a change if present in too great concentration. Conversion of R pneumococci derived from Type II S organisms into Type III pneumococci could be accomplished in almost all instances by means of an extract of Type III organisms. Table I shows the results of a typical experiment.

Further Purification of Extracts

Adsorption on Charcoal.—Attempts were made to separate the active transforming material from the inert cellular debris by the use of various adsorbents. Powdered wood charcoal was found to give

heating to temperatures above 80°C., some extracts have been found active even after an exposure of 10 minutes to a temperature of 90°C. They have been completely inactivated by boiling.

Relatively small amounts of extract have been effective when added to a broth medium containing normal serum or serous fluid. In this medium, R pneumococci, irrespective of their type derivation, have developed and thereafter retained all the type-specific characteristics of the encapsulated S cells from which the extract was prepared.

The specific action of the extracts is discussed with reference to their transforming and antigenic properties.

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out at the bottom of the tube as long as the organisms retained their R characteristics. When S cells developed, however, growth became diffuse throughout the medium. The presence of diffuse growth in the culture medium was presumptive evidence that S colonies would be found later on plating.

TABLE II
Activity of Pneumococcus Extract after Charcoal Adsorption

Activity of <i>Pneumococcus</i> Extract after Charcoal Adsorption							
Tube	Broth	Serum factor (chest fluid)	R strain of <i>Pneumococcus</i> derived from Type:	Specific extract prepared from S pneumococci*		Colonies	Specific agglutination of S colonies
				Type	Amount		
First culture							
1a	1.5	0.5	I	I	0.75	R and S	Type I
2a	1.5	0.5	I	II	0.75	R only	—
3a	1.5	0.5	I	III	0.75	R only	—
4a	1.5	0.5	II	I	0.75	R only	—
5a	1.5	0.5	II	II	0.75	R and S	Type II
6a	1.5	0.5	II	III	0.75	R and S	Type III
7a	1.5	0.5	III	I	0.75	R and S	Type III
8a	1.5	0.5	III	II	0.75	R only	—
9a	1.5	0.5	III	III	0.75	R and S	Type III
10a	1.5	0.5	—	I	1.0†	Sterile	—
11a	1.5	0.5	—	II	1.0	Sterile	—
12a	1.5	0.5	—	III	1.0	Sterile	—
First subculture							
2b	1.5	0.5	From Tube 2a	II	0.75	R only	—
3b	1.5	0.5	From Tube 3a	III	0.75	R and S	Type III
4b	1.5	0.5	From Tube 4a	I	0.75	R and S	Type I
8b	1.5	0.5	From Tube 8a	II	0.75	R and S	Type II
Second subculture							
2c	1.5	0.5	From Tube 2b	II	0.75	R and S	Type II

* Extracts prepared by dissolving S pneumococci in a solution of oxcholate, precipitating in alcohol.

* Extracts prepared by dissolving S pneumococci in a solution of sodium desoxycholate, precipitating in alcohol, redissolving in salt solution, adsorbing on charcoal, and filtering through Berkefeld V candle; Type I extract prepared from Type I pneumococci, Type II from Type II pneumococci, and Type III from Type III pneumococci.

† Controls were prepared using 1.0 and 0.5 cc. quantities of the extract in the original experiment which is shown here much abridged. All controls were sterile.

a motion photomicrographic analysis of the lysis of *Bact. coli* by bacteriophage. Their film, unfortunately, lacks sequence, was not made at a sufficiently rapid rate of exposure and has not been used to provide measurements of the cells.

In order to gain information on these subjects and to obtain an objective measurable record of serial cellular changes, we have taken numerous motion pictures of *Bact. coli* and *B. megatherium* under the influence of bacteriophage. Representative groups of our observations illustrated with photomicrographs of the terminal stages of lysis will be presented in this paper.

Methods and Materials

The photomicrographic apparatus used was an improved form of the one devised by Bayne-Jones and Tuttle (6) in 1927 and now manufactured by the Bausch and Lomb Optical Co. Exposures upon Eastman 16 mm. panchromatic reversal films were made at rates of 2 and 30 per minute and at 8 per second. The light from a 6 volt, 108 watt tungsten filament lamp, 1 m. from the mirror of the microscope, was filtered through a 6 cm. glass cell containing a saturated aqueous solution of quinine bisulfate, neutral density filters and a Wratten B58 green filter transmitting light chiefly in the region 460–600 m μ . These filters were removed for exposures at the rate of 8 per second. No abnormalities of form or growth were attributable to the light. The preparations were under visual inspection at all times.

A magnification of $\times 300$ upon the film, with satisfactory definition, was obtained by using a 1.8 mm. fluorite oil immersion objective and a $\times 3$ negative ocular.

The culture chamber for the preparation to be photographed was made by sealing a glass ring 0.5–0.8 mm. thick on a large No. 2 cover-glass. This was attached to a rectangle of aluminum 1 mm. thick, having a circular opening in the center to permit racking up the substage condenser to compensate for slide thickness. In the well formed by the glass ring a cone of nutrient agar was built up by placing drops of melted agar in the center by means of a capillary pipette. The top of the cone was slightly above the level of the top of the ring. When the agar was firm it was inoculated thinly with bacteria when normal growth was to be observed, or first with bacteria and immediately afterward with a drop of lytic filtrate when bacteriophage action was to be photographed. A No. 1 cover-glass was placed on top of the cone and sealed to the top of the glass ring. This flattened the top of the medium to a table 3–5 mm. in diameter. The bacteria were not compressed but grew here in a single layer. A very thin film of liquid was present between the cover-slip and the agar medium. Distortion and hindrance of growth due to drying occurred occasionally, but was easily detected. The cover-glasses and ring were sterilized in a flame just before use. They were sealed with a mixture

Alcohol was substituted for acetone with equal success. Most of the active material was precipitated by 70 per cent alcohol: all by 100 per cent. The active substance after precipitation in acetone or alcohol was soluble in water. No demonstrable loss in potency resulted from treatment with these reagents as is evidenced by the data presented in Table III.

Properties of the Extract

Resistance to Heat.—The transforming substance was more resistant to heat in extracts freed from much of the extraneous material present in the whole pneumococcus cell and in the crude preparations. Griffith and Dawson both found (1, 3, 6) that heating the intact cells

TABLE III
Activity of Pneumococcus Extract after Precipitation by Acetone

Tube	Broth	Serum factor (chest fluid)	R Pneumo- coccus derived from Type II organisms	Specific extract of Type III pneumo- cocci*	Colonies	Specific agglutination of S colonies
	cc.	cc.		cc.		
1	1.5	0.5	D-39-R	1.0	R and S	Type III
2	1.5	0.5	D-39-R	1.0	R and S	Type III
3	1.5	0.5	D-39-R	0.5	R and S	Type III
4	1.5	0.5	—	1.0	Sterile	—

* Pneumococcus extract prepared by precipitating the charcoal-adsorbed purified extract of S organisms in acetone and extracting the precipitate in distilled water.

to temperatures above 80°C. rendered the bacterial suspensions used in effecting transformations *in vivo*, incapable of inducing changes in type, whereas heating them at lower temperatures, if the exposure was not too long, caused very little decrease in potency. Experiments with purified extracts demonstrated that although these preparations showed a progressive drop in potency on heating above 80°C., nevertheless they were occasionally active after 10 minutes' exposure in the water bath to a temperature as high as 90°C. The effect on the activity of an extract heated for 10 minutes at 70°, 80°, and 90°C., respectively, is evident from the experimental results given in Table IV.

a period of lag, the average largest volume, 3.9 cubic micra, was attained 70 minutes after the start of growth on new medium. After this time, the volume decreased at first rapidly and later more slowly to a relatively constant value of 0.75 cubic micra. The maximum breadth, 1.11μ was attained at 70 minutes and the maximum length, 5.02μ at 90 minutes. The growth rate in bulk of each cell reached a maximum in about $1\frac{1}{2}$ hours after the start and thereafter approached zero rapidly. At first reproduction was associated with large increase in volume. Later, multiplication occurred through fission of cells without increase in volume, subdivisions producing smaller individuals. The average normal generation spans in minutes, indicated by Roman numerals on this graph, were I, 66-90; II, 13-23; III, 15-20; IV, 20; V, 20; VI, 35-40.

Measurements of initial and final dimensions and volume of 46 lysed cells of cultures of *Bact. coli* with bacteriophage are presented in Table I. The complete course of 35 of these cells was known. From them 9 cells which underwent lysis have been selected as representing the various degrees of change observed in the photographs made at the rate of 2 per minute. Measurements of changes in their dimensions and volumes, measurements of the parent of 2 of these cells and other information about them are set forth in Table II. The measurements were made at intervals of 1 minute, but in this table the results are stated at intervals of 10 minutes, or at longer or shorter intervals, according to the occurrence of significant changes.

The period of these observations was approximately the same as that required by a 1-1000 dilution of this specimen of bacteriophage to clear a broth culture. In these experiments the undiluted bacteriophage added to the bacteria on the agar cone did not inhibit growth. The times of lysis after mixture and inoculation and the number of cells lysed (Table I) were as follows: 40-49 min., 2; 50-59 min., 1; 80-89 min., 2; 90-99 min., 2; 120-129 min., 2; 130-139 min., 7; 140-149 min., 4; 150-159 min., 8; 170-179 min., 1; 180-189 min., 5; 190-199 min., 7; 200-209 min., 1; 230-239 min., 1; 250-259 min., 1; 260-269 min., 1. Lysis did not occur before the 40th minute after inoculation. Thereafter, it occurred chiefly in four waves at intervals of 20-40 minutes, with scattering in the last hour of observation. This periodicity of the occurrence of lysis has been noted by

From the data presented in Table IV, it is apparent that pneumococcus extracts became progressively less active after exposure to temperatures above 80°C., and that the loss incurred was approximately in direct proportion to the degree of heating. However, it is also evident that in the present state of purity, an extract occasionally retained sufficient activity to bring about changes even after heating to 90°C. for 10 minutes. Boiling invariably destroyed the activity of the purified extracts.

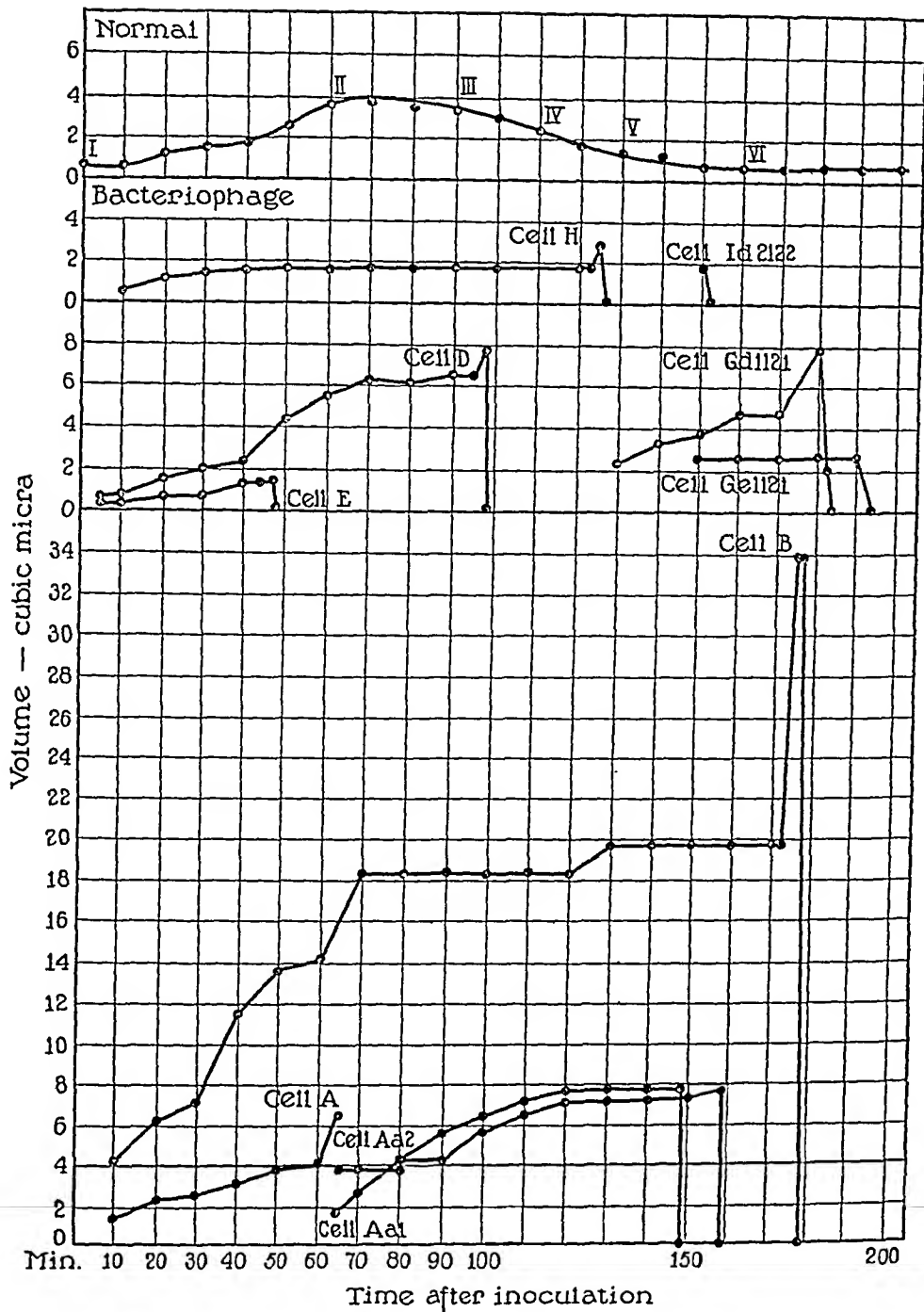
TABLE V
Activity of Purified Pneumococcus Extract after Filtration through Berkefeld W Candle

Tube	Broth	Serum factor (chest fluid)	R organism	Specific extract prepared from Type III S*	Colonies	Specific agglutination of S colonies
First culture						
1a	cc.	cc.	D-39-R†			
2a	1.5	0.5				
3a	1.5	0.5		cc.		
4a	1.5	0.5		1.0	R only	—
5a	1.5	0.5		1.0	R only	—
6a	1.5	0.5		0.5	R only	—
	1.5	0.5	—	0.5	R only	—
				0.25	R only	—
				0.5	R only	—
First subculture						
1b	1.5	0.5	From Tube 1a			
2b	1.5	0.5	From Tube 2a	1.0	R and S	Type III
3b	1.5	0.5	From Tube 3a	1.0	R and S	Type III
4b	1.5	0.5	From Tube 4a	0.5	R and S	Type III
5b	1.5	0.5	From Tube 5a	0.5	R and S	Type III
				0.25	R only	—

* Extract of Type III pneumococci prepared by filtration of adsorbed extract through Berkefeld W candle.

† Strain of R *Pneumococcus* derived from Type II S.

Filtrability.—It was stated in a previous paper (7) that pneumococcus extracts prepared by freezing and thawing the bacterial cells, could be filtered through Berkefeld N candles and still retain their capacity for effecting changes in type. Recent investigations showed that when the purified extract was alkaline in reaction, filtration could be carried out without demonstrable loss of potency, not only



TEXT-FIG. 1. Changes in volume of cells of *Bact. coli*. The upper curve is a graph of averages of volumes of cells of normal cultures at 10 minute intervals during the first 200 minutes of growth. The three sets of graphs below this show volume changes at 10 minute intervals of cells under the influence of bacteriophage. Cell A divided. Cells Aa1, Aa2, B, D, E, Gd1121, Ge12111, H and Id2122 underwent lysis. For data see Table II. Photographs of Cells Aa1, Aa2 and B are reproduced in Figs. 1-8.

specific substance was relatively low, since in most instances the purified extracts reacted specifically in antipneumococcus serum of the homologous type only in dilutions of 1-40, or occasionally 1-80. This indicates a probable concentration of the specific capsular polysaccharide of approximately 0.01 mg. per cc. of extract.

Antigenicity.—It was of interest to learn whether these extracts possessed antigenic as well as transforming properties. It was found that the extracts as prepared were not only active in effecting transformation in type, but also were capable of inducing active immunity in mice treated with the purified preparations. The protocol shown in Table VI illustrates the degree of active immunity induced in mice by repeated intraperitoneal injections of an active extract.

From the results recorded in Table VI, it can be seen that considerable active immunity was induced in mice by three injections, at 3 day intervals, of 0.2 cc. of purified extract of Type I pneumococci. Of 8 mice so treated and inoculated 7 days later with a strain of virulent Type I Pneumococcus, 5 survived an infecting inoculum 100 to 100,000 times greater than that which invariably proved fatal to the normal control animals.

DISCUSSION

Extracts of S pneumococci prepared by the dissolving action of sodium desoxycholate were as active as were the intact cells themselves in causing R forms to assume type-specific characters. Their action was specific, the change in type being selectively determined by the specificity of the extract employed. The active substance or substances in the crude extract inducing the changes could be considerably freed from accompanying impurities by precipitation in alcohol, by charcoal adsorption, and reprecipitation in alcohol or acetone. These procedures seemed not to decrease the potency of the active preparations. In fact, on further purification, the extracts exhibited in most instances increased activity, inducing more prompt transformation.

Despite the fact that the capsular polysaccharide of the Pneumococcus determines its type specificity, it was not possible to correlate the activity of the extracts which stimulate the development of type-specific characteristics in R pneumococci, with the presence of

TABLE II—*Concluded*

Cell No. and generation	Time after inoculation	Length	Breadth	Volume	Volume increase		Duration span
	<i>min.</i>	<i>micra</i>	<i>micra</i>	<i>cubic micra</i>	<i>cubic micra</i>	<i>per cent</i>	<i>min.</i>
D, I	6	1.99	0.66	0.69			93.3
	10	1.86	0.79	0.93	0.27	39.13	
	20	1.99	0.93	1.36	0.67	97.1	
	30	1.59	0.93	1.08	0.39	55.07	
	40	2.66	1.97	2.98	2.29	331.99	
	50	3.25	1.33	4.59	3.90	565.21	
	60	3.32	1.46	5.59	4.90	710.14	
	70	3.46	1.33	4.77	3.08	446.37	
	80	3.58	1.33	4.77	3.08	446.37	
	90	3.46	1.59	6.91	5.22	756.52	
	95	3.46	1.59	6.16	5.47	792.75	
	96	3.46	1.59	6.91	6.22	901.45	
	97	3.46	1.59	6.91	6.22	901.45	
	98	3.72	1.59	7.45	6.76	979.7	
	99	3.72	1.59	7.45	6.76	979.7	
	99.3	3.72	1.59	7.45	6.76	979.7	
E, I	6	1.46	0.66	0.51			42
	10	1.59	0.79	0.79	0.28	56.86	
	20	1.46	0.93	0.99	0.48	94.11	
	30	1.46	0.93	0.99	0.48	94.11	
	40	1.59	0.93	1.08	0.57	111.96	
	48	1.59	0.93	1.08	0.57	111.96	
Gd1121, V	130	3.32	0.93	2.26			51
	140	3.99	0.93	2.72	0.46	20.35	
	150	5.45	0.93	3.71	1.45	64.16	
	160	7.05	0.93	4.79	2.43	107.52	
	170	7.05	0.93	4.79	2.43	107.52	
	180	7.05	1.19	7.92	5.66	250.4	
	181	7.05	1.19	7.92	5.66	250.4	
Ge12111, VI	150	3.86	0.93	2.62			40
	160	3.86	0.93	2.62	0	0	
	170	3.86	0.93	2.62	0	0	
	180	3.86	0.93	2.62	0	0	
	190	3.86	0.93	2.62	0	0	
H, I	10	1.59	0.79	0.79			116
	20	1.79	0.86	1.05	0.26	32.91	
	30	1.99	0.99	1.56	0.77	97.47	
	40	2.13	0.99	1.66	0.87	110.12	
	50	2.39	0.99	1.87	1.08	136.1	
	60	2.66	0.93	1.81	1.02	129.11	
	125	2.66	0.93	1.81	1.02	129.11	
	126	2.66	1.19	2.99	2.20	277.21	
Id2122, V	150	2.66	0.93	1.81			0.03

many others and at one time was correlated with the periodic increase of bacteriophage in the medium, supposedly due to liberation

minutes during the course of preparation, and in sealed glass tubes were completely immersed in the water bath during part of the heating. They were exposed to the action of absolute alcohol for 30 minutes, and were saturated with alcohol of varying strength for several hours. They were treated with charcoal which removed much of the particulate matter, and were finally filtered through Berkefeld V filters. They could even be heated to 90°C. for short periods, or passed through Berkefeld W filters and still remain active. Controls of sterility were exceedingly rigid. The extracts were injected in large amounts into mice without any untoward effects. All cultures of the extracts and of animals sacrificed at various intervals after the injection of active material, were sterile.

The exact nature of the active material in these extracts still remains to be determined. That it acts as a specific stimulus to the R cells which have potentially the capacity of elaborating the capsular polysaccharides of any one of the several types of pneumococci seems clear.

SUMMARY

Pneumococcus extracts highly active in inducing the *in vitro* transformation of the specific types of Pneumococcus have been prepared by dissolving S cells with sodium desoxycholate, precipitating the dissolved material in alcohol in which the bile salt remains soluble, and extracting the precipitate in salt solution. Further purification of these active extracts has been attained by the removal of considerable inactive material by charcoal adsorption and by reprecipitation of the adsorbed extract in alcohol or acetone. The importance of using young cultures for extraction, and of preventing autolysis during the preparation of the extracts, is emphasized.

Extracts prepared by the method described have been filtered through Berkefeld Candles (V, N, and W) without appreciable loss in activity, provided the reaction of the extract was slightly alkaline at the time of filtration. The purified and filtered extracts are water-clear, and sterile by rigid cultural and animal tests. They have been heated to temperatures of 60°C. for 30 minutes without appreciable loss in their capacity to induce specific changes in type. And although they have generally shown definite decrease in potency after

averages to answer questions relating to the increase in volume of cells of *Bact. coli* undergoing lysis by bacteriophage. The average volume of the 46 lysed cells (Table I) was 4.07 cubic micra. This is only slightly greater, 0.17 cubic micra (4.36 per cent), than the average maximum volume of normal cells. When, however, these cells are compared with cells of the same generation and with cells in cultures of the same age as these at the time of lysis it is seen that while a few are the same size as that of the average for their generation and age, most of them are from 1.5–3 times as large as normal cells of the same generation and from 2–18 times as large as cells in normal cultures of the same age. The comparison is distorted by the persistence of first generation cells through a period at which the normal culture would have passed the sixth generation. As we shall show, there is apparently a relationship between generation and capacity to enlarge. The increase in volume of the 35 cells whose histories were known was from 0–979 per cent; 11 cells increased by 100–979 per cent; 24 increased by 0–87 per cent. The 24 cells which did not double their volumes during this phase of bacteriophage action included 14 sixth generation cells. All were larger than cells of the same generation in normal cultures. The progress of this phase of increase in size is shown in Figs. 1–8.

The relationship of the degree of increase in volume to the generation of the cell can be obtained from Table I. First generation cells, with 2 exceptions, increased volume by 111–979 per cent. One of these, Cell B, attained enormous proportions. Second generation cells, with 2 exceptions, increased from 120–181 per cent; third generation cells from 19–131 per cent; fifth generation cells from 5–250 per cent; sixth generation cells from 0–87 per cent, with the mean increase about 20 per cent. Both the absolute sizes and the percentage increase in volume of the first and second generation cells were much greater than the volumes and increase in volume of the cells of the later generations. It is apparent, therefore, that the capacity for volume enlargement by swelling is greater in cells of the first three generations than in those of later generations, without regard to their duration or prolonged age at the time of lysis.

The final stage of lysis occupies so brief an interval that the lysed cells seem to flash from view. By making exposures at the rate of 8

CHANGES IN THE SHAPE AND SIZE OF BACTERIUM COLI
AND BACILLUS MEGATHERIUM UNDER THE INFLU-
ENCE OF BACTERIOPHAGE—A MOTION PHOTOMI-
CROGRAPHIC ANALYSIS OF THE MECHANISM
OF LYSIS

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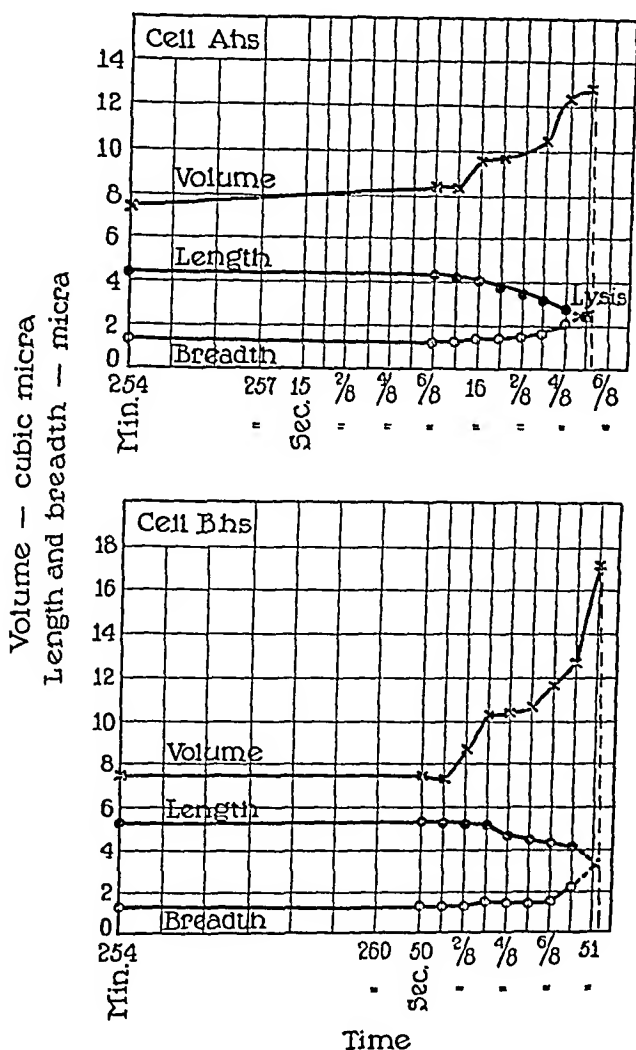
PLATES 25 TO 27

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Outlines of the changes in shape and size of bacteria under the influence of bacteriophage have been drawn from numerous visual and photographic records, and this composite material has given some insight into the mechanism of the lytic process. These observations, amply reviewed by Bronfenbrenner (1), do not require recapitulation here. Most observers have confirmed d'Herelle's (2) statement that Gram-negative bacteria in a moist medium enlarge by swelling and eventually burst. Whether Gram-positive bacteria swell or burst is still an open question, as shown by the contradictory evidence in the recent papers of Bronfenbrenner (3) and Krueger and Northrop (4). The time intervals of the morphological changes preceding lysis, the relation of these changes to stages of growth and the time occupied by the final bursting or disintegration have not been precisely determined. The amount, character, distribution and persistence of the residue remaining after lysis have not been adequately described. Very few measurements of dimensions of cells or of time intervals have been presented in reports on the bacteriophagic process. In their lack of quantitative data on serial changes in cells and their residues, the available records are deficient in the material needed for the construction of generalizations.

Recognizing this deficiency, Bronfenbrenner, Muckenfuss and Hetler (5) were the first to attempt to supply the required data by making

there were band-like enlargements across the transverse axis of the cells, and the ends of the cells appeared to be less swollen proportionately than the central regions.



TEXT-FIG. 2. Changes in dimensions and volumes of Cells Ahs and Bhs of culture of *Bact. coli* during the terminal stages of lysis by bacteriophage. For data see Table III. Photographs of these cells are reproduced in Figs. 11-33.

Photographs of Cell B, Figs. 1-8, and Cells Ahs and Bhs, Figs. 11-33, show a more or less round or oval clear area in the central region of the cells. This appeared first when swelling began and con-

of beeswax and paraffin which was carefully kept from any contact with the medium.

The cultures were incubated at $37 \pm 0.1^\circ\text{C}$. in box with circulating air completely surrounding the microscope.

The developed films were studied by rapid projection and by the projection of single frames enlarged 25 times, giving a $\times 7500$ magnification of the organisms. These methods of projection and measurement, together with a discussion of the errors of measurement, have been presented in recent papers by Bayne-Jones and Adolph (7-9). The errors of measurement were estimated to be somewhat less than 10 per cent of the actual dimensions of the cells. Regular series of measurements were obtained by practice.

The formula for the volume of a cylinder, $\pi/4 \times \text{length} \times (\text{breadth})^2$ was used for most of the calculations of the volumes of the cells of both *Bact. coli* and *B. megatherium*. For some of the ellipsoidal and spherical cells of *Bact. coli* near the end of lysis $\pi/5 lb^2$ and $4/3 \pi r^3$ were used to calculate the volumes.

The strain of *Bact. coli* marked S because of its sensitivity to bacteriophage, and the bacteriophage for this organism were received from Dr. George H. Smith in 1906. The culture of *B. megatherium*, Kral strain, was received from Dr. P. B. Cowles in 1930, together with a specific bacteriophage filtrate described by Cowles (10). The titers of these lytic filtrates by dilution were respectively 10^{-9} and 10^{-6} .

The bacteria were maintained on 2 per cent agar containing 2 per cent peptone, 0.5 per cent NaCl, with a reaction of pH 7.4. The same medium was used for the agar cones in the culture chambers. Transfers to the agar cones were made from cultures previously incubated for 16-24 hours at 37°C .

The lytic filtrates were used undiluted on the agar cones in the culture chambers. For the hanging drop cultures in peptone water, the bacteriophage filtrate lytic for *Bact. coli* was diluted 1-10.

Observations on the Lysis of Bact. coli

It is well known that the sizes of bacteria are different at different ages of the culture. This variability must be taken into consideration in deciding whether a particular cell in a culture with bacteriophage is larger than a cell of the same generation and age in a normal culture. As a basis for this comparison we have made and used measurements from motion picture films of normal cultures grown by us under the same conditions as those existing in our cultures with bacteriophage. These data and their analysis have been published by Bayne-Jones and Adolph (8). Material from that paper with sets of measurements made especially as controls for this investigation are summarized in the upper graph of Text-fig. 1. This graph shows the progression of changes of volume during the first 200 minutes of growth. After

of bacteriophage are those relating to Cell G and its descendants. We have records of measurements of Cell G and 46 of its descendants. This family flourished and died out in an area of active bacteriophagic lysis during a period of 230 minutes. Generation times were as follows: I, 65 min., II, 20 min., III, 15 min., IV, 15–20 min., V, 15–50 min., VI, undetermined. The generation spans up to the fourth generation were normal. Abnormality evidenced by lengthening of the generation span did not occur until the fifth generation was reached. The first 3 cells of this family to undergo lysis were members of the fifth generation. Twelve remaining members of this generation could be observed. All except 2 of them divided in from 15–20 minutes. Seventeen out of the possible 26 members of the sixth generation could be seen and measured. Of these 14 underwent lysis in 38–60 minutes, 2 divided after 50 minutes and 1 had neither divided nor dissolved 185 minutes after its formation, when the camera was stopped. The volumes of the cells in the first three generations were somewhat but not significantly larger than those of normal cells of these generations. The records show that while bacteriophage inhibits the multiplication of some first generation cells, it may affect the size but not the reproductive processes of other cells. It seems probable that bacteriophage was absorbed by members of at least the second and third generations of this G family and was passed on to the descendants at times of fission until lysis ended the lives of the individuals.

Observations on the Lysis of B. megatherium

Measurements of changes in dimensions and volumes of cells of normal cultures of *B. megatherium* must be used for comparison with the dimensions and volumes of cells of this species under the influence of bacteriophage. Our comparisons were made with measurements of normal cells at half-hour intervals during 14 hours of growth, under the conditions prevailing in these experiments. These measurements were averages of those published by Adolph and Bayne-Jones (9) and of those made in immediate connection with this motion photomicrographic study of the lysis of *B. megatherium*. The progression of changes in volume are shown in the upper graph of Text-fig. 3. Average length of cells of *B. megatherium* starting from 2.6μ passed

TABLE I
Initial and Final Dimensions and Volumes of 46 Cells of *Bact. coli* Lysed by Bacteriophage

Times are given in minutes after inoculation of the medium. Volume = $\pi/4$ length x (breadth)² in all calculations except those marked*. Volume was calculated as for a sphere, $V = 4/3\pi r^3$. In these cases

Cell No. and generation	Time of birth <i>min.</i>	Time of lysis <i>min.</i>	Span <i>mic.</i>	Length		Breadth		Volume		Volume increase <i>per cent</i>
				Initial <i>micra</i>	Final <i>micra</i>	Initial <i>micra</i>	Final <i>micra</i>	Initial <i>cubic micra</i>	Final <i>cubic micra</i>	
Aa1, II	66	150	84	2.93	5.32	0.93	1.33	1.99	7.34	268.84
Aa2, II	66	156	90	2.99	5.32	0.93	1.33	3.35	7.39	120.6
B, I	6	178	172	6.12	14.36	0.93	1.73	4.21	33.75	701.66
Ca1, II	10	80	70	1.86	3.72	0.93	1.46	1.26	6.25	392.2
Ca2, II	10	132	122	1.78	2.26	0.79	1.19	0.89	2.53	181.1
D, I	6	99	93	1.99	3.72	0.66	1.59	0.69	7.45	25.2
E, I	6	48	42	1.46	1.59	0.66	0.93	0.51	1.08	979.7
F, I	6	48	25	1.46	1.59	0.66	0.93	0.79	0.99	111.96
Gd1111, V	130	155	25	2.92	3.06	0.93	0.93	1.99	2.08	5.0
Gd1112, V	130	155	25	2.92	3.06	0.93	0.93	1.99	2.08	5.0
Gd1121, V	130	181	51	3.32	7.05	0.93	1.97	2.26	7.92	250.4
Ge11221, VI	150	188	38	3.06	3.79	0.93	1.06	2.08	3.66	62.0
Ge11222, VI	150	188	38	3.06	3.88	0.93	0.93	2.17	2.62	20.7
Ge12111, VI	150	190	40	3.86	3.86	0.93	0.93	2.62	2.62	0
Ge12112, VI	150	190	40	3.86	3.86	0.93	0.93	1.85	2.62	0
Ge12121, VI	150	190	40	3.86	3.86	0.93	0.93	2.62	2.62	0
Ge12122, VI	150	195	45	3.86	3.59	0.93	0.93	1.85	2.62	31.9
Ge21111, VI	150	210	60	3.13	3.39	0.93	0.93	2.13	2.30	8.5
Ge21112, VI	150	230	80	3.06	3.46	0.93	0.93	2.08	2.35	12.9
Ge21122, VI	150	187	47	2.93	3.33	0.93	1.19	1.63	3.04	80.9
Ge21211, VI	140	191	51	3.39	3.06	0.93	1.67	2.05	24.5	24.5
Ge21212, VI	140	195	55	3.39	3.86	0.93	1.63	1.99	22.1	22.1
Ge22211, VI	140	200	60	2.46	3.06	0.93	1.63	2.99	277.21	—
H, I	140	195	55	2.46	3.06	0.93	1.67	2.05	24.5	24.5
Id2111, V	10	126	116	1.59	2.67	0.79	1.19	0.79	2.06	33.2
Id2112, V	150	151	1	2.66	—	—	—	—	—	—
Ja1, II	85	130	45	4.12	5.72	0.79	0.79	2.19	2.93	33.2
Ja2, II	85	130	45	4.12	5.72	0.79	0.79	2.19	2.93	33.2
Kb11, III	100	125	25	2.67	3.19	0.79	1.06	3.07	131.55	301.4
Kb12, III	100	135	35	2.67	3.46	0.79	1.06	3.07	131.55	301.4
M, I	15	85	70	1.46	2.67	0.79	1.06	3.07	131.55	301.4
N, I	15	85	70	1.46	2.67	0.79	1.06	3.07	131.55	301.4
O, I	15	85	70	1.46	2.67	0.79	1.06	3.07	131.55	301.4
Ahs, ?	?	257	?	4.52	2.77	1.46	7.52	12.94*	444.7	72.04
Bhs, ?	?	260	?	5.45	3.2	1.33	7.58	2.54	124.67	13.4
Ibr, ?	?	150	?	1.99	2.26	1.19	2.48	2.48	35.4	0
IIbr, ?	?	142	?	2.79	2.79	1.06	3.07	3.07	16.4	—
IIIbr, ?	?	141	?	2.13	2.26	1.06	3.07	3.07	16.4	—
IVbr, ?	?	140	?	3.46	3.46	1.06	3.07	3.07	16.4	—
Vbr, ?	?	142	?	2.79	2.79	1.06	3.07	3.07	16.4	—
VIbr, ?	?	140	?	2.79	2.79	1.06	3.07	3.07	16.4	—
VIIbr, ?	?	135	?	2.79	2.79	1.06	3.07	3.07	16.4	—
VIIIbr, ?	?	145	?	2.66	2.99	1.06	3.07	3.07	16.4	—
IXbr, ?	?	155	?	3.46	3.46	1.06	3.07	3.07	16.4	—
	?	135	?	3.06	—	—	—	—	—	—

TABLE IV

Dimensions and Volume of Cells of B. megatherium under Influence of Bacteriophage

Cells placed in view 145 minutes after inoculation of the medium. These are shown in Figs. 34-43. Generations estimated to be III and IV. Volume = $\pi/4$ length \times (breadth)². Lysis complete at time indicated in line with dashes. Cells too indistinct for measurement during last minute.

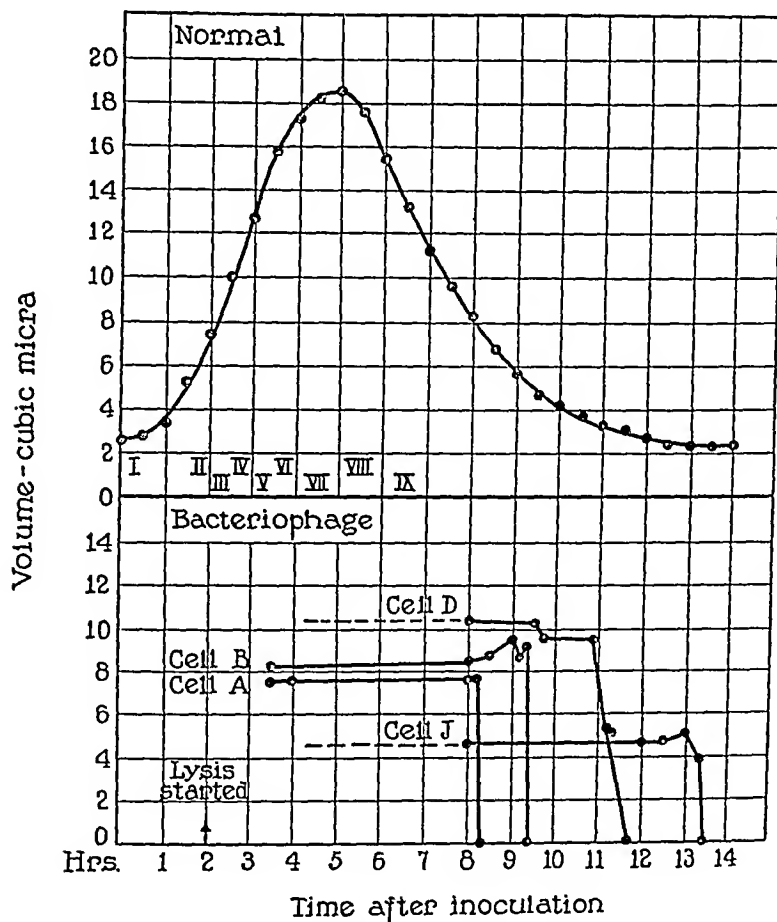
Cell No.	Time after inoculation	Duration under observation	Length	Breadth	Volume	Volume increase	
	min.	min.	micra	micra	cubic micra	cubic micra	per cent
A	481	351	5.59	1.33	7.75		
	496		5.59	1.33	7.75	0	0
	496.1		—	—	—	—	—
B	481	421	5.72	1.39	8.62		
	495		5.91	1.33	8.21	-0.41	-5.1
	525		5.91	1.39	8.91	0.39	4.7
	555		5.91	1.46	9.94	1.32	15.31
	564		5.1	1.46	8.5	-0.15	-1.72
	566		5.32	1.33	9.42	0.80	14.80
	566.5		—	—	—	—	—
C	481	433	3.99	1.33	5.54		
	495		3.99	1.33	5.54	0	0
	566		3.99	1.33	5.54	0	0
	578		3.19	1.19	3.58	-1.96	-35.37
	578.5		—	—	—	—	—
C1	481	438	3.99	1.33	5.54		
	525		3.99	1.33	5.54	0	0
	583		3.99	1.33	5.54	0	0
	583.1		—	—	—	—	—
D	481	549	6.78	1.39	10.22		
	581		6.78	1.39	10.22	0	0
	582		6.78	1.33	9.42	-0.80	-7.80
	694.5		5.85	1.06	5.19	-5.03	-49.20
	694.6		5.72	1.06	5.07	-5.15	-50.40
	694.7		—	—	—	—	—
E	481	526	3.99	1.46	6.72		
	495		4.19	1.46	7.04	0.32	4.7
	525		4.52	1.46	7.59	0.87	12.8
	670		3.72	1.46	6.26	-0.46	-6.8
	671		—	—	—	—	—
F	481	531	4.59	1.46	7.71		
	525		4.79	1.46	8.04	0.33	4.2
	674		4.26	1.46	7.15	-0.56	-7.2
	674.7		3.86	1.46	6.48	-1.23	-15.9
	676		—	—	—	—	—
G	481	443	2.67	1.33	3.7		
	495		2.79	1.33	3.88	0.18	4.03
	525		3.59	1.33	4.93	1.28	34.6
	555		3.99	1.33	5.55	1.85	50.0
	584		3.72	1.33	5.17	1.47	40.0
	588		—	—	—	—	—

TABLE II
Dimensions and Volumes of 10 Cells of Cultures of *Bact. coli* under Influence of Bacteriophage

Measurements and calculations recorded at 10 minute intervals. Curves of volume changes of these cells shown in Text-fig. 1. Volume = $\pi/4$ length \times (breadth)². Cell A divided. All other 9 cells underwent lysis.

Cell No. and generation	Time after inoculation	Length	Breadth	Volume	Volume increase		Duration span
					cubic micra	per cent	
A, I	min.	micra	micra	cubic micra			min.
	10	2.46	0.93	1.67	0.78	46.76	56
	20	2.79	1.06	2.45	0.96	57.48	
	30	2.99	1.06	2.63	3.35	100.0	
	40	3.39	1.13	3.51	1.68	110.18	
	50	3.99	1.06	3.51	1.84	152.09	
Aa1, II	60	4.72	1.06	4.21	2.54		
	66	5.72	1.06	6.41	4.74	283.83	
	70	2.93	1.19				
	80	3.52	0.93	1.99			
	90	4.65	1.06	3.09			
	100	4.92	1.06	4.12	1.10	55.27	84
Aa2, II	110	5.32	1.19	4.21	2.13	107.03	
	120	5.05	1.19	5.96	2.22	111.56	
	130	5.05	1.19	5.66	3.97	199.49	
	140	5.32	1.33	5.66			
	150	5.32	1.33	7.34	5.35	268.84	
	156	5.32	1.33	7.34	5.35	268.84	
B, I	66	2.99	1.19				
	70	3.26	1.19	3.35			
	80	4.39	1.06	3.65	0.30	8.95	90
	90	5.05	1.19	3.86	0.51	15.22	
	100	4.92	1.33	6.84	2.31	68.95	
	110	5.12	1.33	7.12	3.49	104.18	
	120	5.05	1.33	7.02	3.77	112.53	
	130	5.05	1.33	7.02	3.67	109.55	
	140	5.05	1.33	7.02			
	150	5.18	1.33	7.21			
	154	5.32	1.33	7.39	3.86	115.22	
	156	5.32	1.33	7.39	4.04	120.60	
	10	6.12	0.93	4.21	4.04	120.60	
	20	7.85	1.06	6.90			
	30	8.58	1.06	7.62	2.79	63.89	172.4
	40	10.44	1.19	11.72	3.41	81.0	
	50	12.10	1.19	13.55	7.51	178.38	
	60	12.63	1.19	14.15	9.34	221.87	
	70	13.43	1.33	18.64	9.94	236.09	
	120	13.43	1.33	18.64	14.43	342.73	
	130	14.29	1.33	19.72	15.51	368.41	
	140	14.36	1.33	19.93	15.72	373.39	
	174	14.36	1.46	24.13	19.92	473.15	
	175	14.36	1.59	28.72	24.51	552.18	
	176	14.36	1.66	30.81	26.60	631.85	
	177	14.36	1.73	33.73	29.54	701.66	
	178	14.36	1.73	33.75	29.54	701.66	
	178 4	14.36	1.73	33.75	29.54	701.66	

in the third and fourth generation at the time when reproduction ceased. It is seen from Table IV that the approximate duration spans of these cells was from 496-791 minutes. They overlapped without fission all the generations that would have occurred in a



TEXT-FIG. 3. Changes in volume of cells of *B. megatherium*. The upper curve is a graph of averages of volumes of cells in normal cultures at half-hour intervals during 14 hours of growth. The lower part of the chart shows graphs of volume changes of Cells A, B, D and J, which underwent lysis. For data see Table IV. Photographs of these cells are reproduced in Figs. 34-43.

normal culture from about the 2nd or 3rd to the 13th hour. We do not know how many generations would have occurred normally in this period, but know that normal cultures of this species pass the ninth generation before the 7th hour of growth.

of the lytic agent from the disintegrated cells. From more recent studies of the production of bacteriophage without lysis it has been suggested that disintegration of the cells is not essential for the liberation of bacteriophage. Before the conclusion that lysis is not necessary for the increase of bacteriophage in the medium is accepted the question should be investigated by direct observation of the cells.

This periodicity of lysis might be related to generations of bacteria in the cultures. Our observations supply some information on this point. The duration spans of the 35 cells whose whole course was known (Table I) were as follows: 0-9 min., 2; 10-19 min., 0; 20-29 min., 3; 30-39 min., 4; 40-49 min., 10; 50-59 min., 4; 60-69 min., 2; 70-79 min., 3; 80-89 min., 2; 90-99 min., 2; 116 min., 1; 122 min., 1; 172 min., 1. In these figures there is no evidence of periodicity of lysis. The durations of the cells were scattered over the whole period. The frequency of durations increased gradually to 10 in the period 40-49 minutes and decreased gradually after that time. Among these 35 cells there were, by chance, no fourth generation cells and a disproportion of sixth generation cells. The duration spans of 8 first generation cells were from 37-172 minutes. These cells which never underwent fission had the longest duration spans before lysis. The duration spans of second generation cells were from 45-122 minutes; of third generation cells from 25-35 minutes; of fifth generation cells from less than 1 minute to 51 minutes; of sixth generation cells from 38-80 minutes. The cells were obviously different in their resistance to lysis, some surviving longer than others after their contact with bacteriophage. Two cells were observed to undergo lysis less than 1 minute after visible fission. The data on these groups of cells failed to show that there was a correlation between periodicity of lysis and periodicity of fissions.

Our observations indicate that the volume changes of *Bact. coli* under the influence of bacteriophage pass through at least two phases. The first phase is a slow enlargement or maintenance of a definite size. The second is a rapid explosive enlargement. It will be convenient to deal with these phases separately. Measurements from motion pictures made at the rate of 2 per minute are adequate for analysis of the first phase. The tables and graphs demonstrate at a glance that it would be futile to attempt to use

nous and appeared to be composed of amorphous material from the bacterial substance together with refractile granules which were visible before lysis. There was no indubitable evidence of hollow shells of cell walls remaining after disintegration of the internal substance of the cell.

In the microscopic observation of lysis of *Bact. coli* we were always able to find foci of bacteriophagic activity. What, no doubt, would appear to the naked eye to be a plaque can be seen under the microscope to be composed of scattered cells undergoing lysis in different groups separated by 10 to 20 μ . Lysis spread to contiguous cells. This spreading of the action of bacteriophage was seen most clearly in the bands of cells of *B. megatherium* shown in Figs. 34-43. Lysis began at the point marked "Sp" in Text-fig. 6. By grouping the cells in the order of their disintegration, it is seen that lysis spread first along the Chain A, B, C, C1. Then it started in Cell G and spread through the Group G, G1, H and H1. From a third focus it spread next through the Group D, E and F. The last group to be affected were Cells K, K1, M, N, O and O1. In this small field there were four foci of lysis. From each focus lysis traveled through a group of contiguous and related cells. The spread was partly by contact, but also definitely along paths of genetic relationships.

DISCUSSION

It is obvious that these visual and photographic records cannot explain the whole mechanism of lysis and give no direct information about the nature of bacteriophage. Nevertheless, they record in quantitative terms phenomena which must be taken into account by any theory. They provide a basis for critical comments on prevailing groups of theories.

Since many cells of *Bact. coli* and all the dissolved cells of *B. megatherium* underwent lysis without enlargement, it is apparent that swelling is not an invariable or necessary antecedent to lysis. These observations, therefore, are opposed to d'Herelle's theory that bacteriophage distends the cells it dissolves.

Our observations were not capable of giving any evidence upon the possibility of the production of minute invisible forms from the lysed cells. The process of lysis looked like a bursting or disintegration,

per second we succeeded in obtaining a series of photographs of the last stages of disruption of 2 cells of *Bact. coli* (Figs. 11-33). Measurements from these films and graphs of the data are presented in Table III and Text-fig. 2.

In this last phase of lysis the shape of the swollen cell changed from a cylinder to an oval ellipsoidal form and finally approximated a

TABLE III
Changes in Dimensions and Volume of 2 Cells of *Bact. coli* in the Final Stage of Lysis by Bacteriophage

Measurements from motion picture film with exposures at the rate of 8 per second

Cell No.	Time after inoculation		Length micra	Breadth micra	Volume		Volume increase cubic micra per cent	Remarks, formulae for volume
	min.	sec.			cubic micra			
Ahs	253	18	4.52	1.46	7.52	—	—	$V = \pi/4 lb^2$
	254	18	4.52	1.46	7.52	—	—	
	257	15 $\frac{5}{8}$	4.19	1.59	8.33	0	0	
	16	15 $\frac{5}{8}$	4.12	1.59	8.33	0.81	10.77	
	16 $\frac{1}{2}$	15 $\frac{5}{8}$	4.06	1.73	9.73	0.81	10.77	
	16 $\frac{3}{4}$	16 $\frac{1}{8}$	3.86	1.73	9.73	2.21	29.39	
	16 $\frac{5}{8}$	16 $\frac{3}{8}$	3.59	1.93	(10.0)	2.21	29.39	
	16 $\frac{3}{4}$	16 $\frac{5}{8}$	3.26	2.26	10.47	2.48	33.0	
	16 $\frac{7}{8}$	16 $\frac{3}{4}$	2.93	2.6	12.45	2.95	39.23	
	16 $\frac{7}{8}$	16 $\frac{7}{8}$	(2.77)	(2.77)	12.94	4.93	65.56	
Bhs	253		5.45	1.33	7.58	5.42	72.07	$V = \pi/5 lb^2$
	254		5.45	1.33	7.58	—	—	$V = \pi/5 lb^2$
	257		5.19	1.33	7.58	0	0	$V = 4/3\pi r^3$ Burst
	260		5.19	1.33	7.23	0	0	$V = \pi/4 lb^2$
		50	5.12	1.46	8.65	—	—	
		50 $\frac{1}{8}$	4.79	1.6	10.19	1.07	13.85	
		50 $\frac{1}{4}$	4.52	1.66	10.28	2.61	34.43	
		50 $\frac{3}{8}$	4.39	1.73	10.61	2.70	35.62	
		50 $\frac{1}{2}$	4.26	1.86	11.93	3.03	39.97	
		50 $\frac{3}{4}$	(3.2)	(3.2)	12.59	4.35	57.37	
					5.01	66.08		$V = \pi/5 lb^2$
					9.45	124.67		$V = 4/3\pi r^3$ Burst

sphere. The length decreased while the breadth increased until both dimensions became approximately equal. The volume, calculated by appropriate formulae indicated by the photographs, increased rapidly and relatively enormously during the fractions of seconds of this final stage. The cells burst. The period of this explosive enlargement was $\frac{1}{2}$ second in the case of Cell Ahs and $\frac{7}{8}$ second in the case of Cell Bhs. While the changes in shape were on the whole regular,

have no experimental data to support this hypothesis, but our measurements of cellular changes and pictures of the cellular debris suggest that surface tension changes may be important in the mechanism of lysis.

SUMMARY AND CONCLUSIONS

This paper contains the records of a motion photomicrographic investigation of the lysis of *Bact. coli* and *B. megatherium* by bacteriophage. The bacteria mixed with bacteriophage were grown on moist nutrient agar in small culture chambers on the stage of a microscope in an incubator maintained at 37°C. The apparatus used permitted continuous inspection of the preparations. Photographs were made at the rates of 2 and 30 per minute and at the rate of 8 per second during the terminal stage of lysis of *Bact. coli*. The accurately timed films were studied by rapid projection and by the projection of single frames. Measurements of dimensions of cells, calculations of volumes, information on generations, generation times and duration spans are presented in the tables. Similar information on normal cultures grown and photographed in the same way is furnished for comparison. Groups of serial photographs are reproduced in the plates to illustrate the special features observed.

These observations seem to us to warrant the following conclusions:

1. Enlargement or swelling of the cells of *Bact. coli* usually, but not always, precedes lysis. Some of the enlargement is an expression of increase of cell substance and is not altogether due to imbibition of water. Cells of early generations of *Bact. coli* enlarge to greater absolute and relative proportions than cells of later generations. Enlargement does not occur before lysis in *B. megatherium*.

2. The terminal stage of lysis of *Bact. coli* is explosive, occupying $\frac{1}{2}$ to $\frac{7}{8}$ second. The terminal stage of lysis of *B. megatherium* is a slow disintegrative process, extending over 2–10 minutes.

3. Bacteriophage inhibits fission of some cells, but does not stop the reproduction of other cells in contact with it. The genealogical records of six generations of cells of *Bact. coli* and of two generations of cells of *B. megatherium* indicate that bacteriophage may be transmitted through parents to the offspring which ultimately undergo lysis.

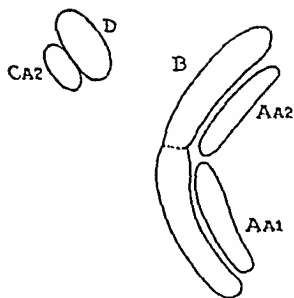
tinued to the end. Its shape did not change greatly during the swelling of the cells. We are unable to state whether this is a "pore" or vacuole or an optical effect of focus giving a bright spot at the top of a convex surface. We are inclined to interpret this appearance as an optical effect rather than an indication of a physical vacuole.

Rapid projection of the films showed a scattering of cellular debris at the moment when a cell burst. This debris was flung to a distance several times the width of the cell. The debris was amorphous and contained a few granules if any were previously present in the cells. The residue had the appearance of gelatinous material. No cell envelope was visible among the masses or blocks of bacterial substance. It was suggested by an expert microscopist that an effect like that seen in these photographs might have been produced by minute compressions or fractures in the agar. To test this point, we photographed cells lysed in peptone water. Here also a similar type of debris remained visible after bursting of the cells, as shown in Figs. 9 and 10. We are of the opinion that the pattern of the debris, its appearance of being projected from the region of the bursting cell, its amount and its visibility in a liquid medium, indicated that these appearances were produced by particles of bacterial substance and were not the optical effects of physical changes in the medium. The debris gradually decreased, apparently by solution or dispersion, but it did not altogether disappear. It could be recognized 10-12 hours after the completion of lysis.

The data presented in Table I give some information upon the effect of bacteriophage on reproduction by *Bact. coli*. All the first generation cells which underwent lysis began to grow, increased in volume by assimilation or swelling to various degrees and persisted for different intervals up to 178 minutes in a medium containing undiluted bacteriophage. With the exception of Cell B, which showed partial fission, none of these cells divided, although fission would have been expected in normal cells of their relative size and age. Evidently, bacteriophage hindered multiplication of cells which retained some capacity for growth in volume and a great capacity for abnormal enlargement. Other cells in the midst of bacteriophage grew and passed through three or four generations before any appearance of abnormality occurred. The best data we obtained on this phase of the action

EXPLANATION OF PLATES

PLATE 25



TEXT-FIG. 4. Diagram of cells of *Bact. coli* shown in Figs. 1-8.

FIGS. 1-10. Serial photographs of changes in size and shape of cells of *Bact. coli* under the influence of bacteriophage. For diagram of cells see Text-fig. 4. Enlargements from motion picture film made Dec. 9, 1931. Exposures at rate of 2 per minute. $\times 2000$.

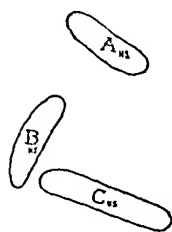
FIGS. 1-8. *Bact. coli* with bacteriophage in culture on agar.

FIGS. 9-10. *Bact. coli* with bacteriophage in a drop of peptone water.

Times after inoculation are as follows: Fig. 1, 60 min.; Fig. 2, 120 min.; Fig. 3, 146 min.; Fig. 4, 150 min.; Fig. 5, 155 min.; Fig. 6, 155 min. 30 sec.; Fig. 7, 178 min. 10 sec.; Fig. 8, 178 min. 40 sec.; Fig. 9, 135 min.; Fig. 10, 156 min.

The great swelling of cells is clearly shown, particularly of Cells Aa1, Aa2 and B. The cellular debris, scattered when the cells burst, remains visible both on agar and in peptone water.

PLATE 26



TEXT-FIG. 5. Diagram of cells of *Bact. coli* shown in Figs. 11-33.

FIGS. 11-33. Serial photographs of final stages of lysis of Cells Ahs and Bhs in culture of *Bact. coli* with bacteriophage on agar medium. Exposures at rate of 8 per second. Enlargements from motion picture film made Mar. 11, 1932. $\times 2000$. For diagram of cells see Text-fig. 5.

Times after inoculation are as follows: Fig. 11, 253 min. $1\frac{5}{8}$ sec.; Fig. 12, 257 min. $1\frac{5}{8}$ sec.; Figs. 13-22, thereafter at intervals of $\frac{1}{8}$ sec.; Fig. 23, 257 min. $17\frac{1}{8}$ sec.; Fig. 24, 260 min. 50 sec.; Figs. 25-33, thereafter at intervals of $\frac{1}{8}$ sec.

The photographs show the rapid change from the cylindrical through ellipsoidal to spherical form occurring during the last seconds of lysis. Both of these cells show bulging in the region of the transverse axis. This type of lysis by bursting occupies from $\frac{1}{2}$ to $\frac{7}{8}$ second. Cellular debris remains visible after lysis.

The photographs show more or less round clear areas in the central regions of the cells. These may be vacuoles, but are probably to be interpreted as optical effects produced by a convex surface.

Granules in the medium and specks due to enlarged grains in the films are readily distinguishable from bacterial debris, especially when the films are viewed by rapid projection.

through a maximum of 12μ at 5 hours and thereafter decreased gradually to the original value. Breadth increasing from 1.11μ to a maximum of 1.41μ at 4 hours also gradually returned to its first value. Volume increased relatively rapidly from 2.48 cubic micra to 18.48 cubic micra at approximately the eighth generation at 5 hours and decreased at first rapidly and then more slowly to its original value. Some normal cells were larger than the average, reaching 24 cubic micra in volume; others were as small as 2 cubic micra. Conditions of culture and heredity or peculiarity of families of cells influence these sizes. The average generation spans in minutes, indicated by Roman numerals on the graph, were as follows: I, 77; II, 30; III, 20; IV, 30-33; V, 30-40; VI, 40; VII, 50-60; VIII, 60; IX, 60-70.

After *B. megatherium* was mixed with bacteriophage on nutrient agar in the manner described in the first section of this paper, growth in size and multiplication proceeded normally for approximately 2 hours. Lytic action usually became manifest at about that time and continued during the succeeding 12 or more hours. The process was slow in beginning and slowly brought about disintegration of the cells. The measurements obtained from our motion photomicrographs, using films made with exposures at the rate of 2 per minute until the commencement of lysis and thereafter at the rate of 30 per minute provide material that can be used to answer the question as to whether or not the cells of *B. megatherium* enlarge or swell before or during lysis. Measurements of dimensions and calculations of volumes of 18 cells which underwent lysis are presented in Table IV. We have measurements of these cells at 10 minute intervals, but include only the instants of change in this table. The progression of volume changes of 4 representative cells are shown in the graphs in the lower half of Text-fig. 3. Photographs of these cells at different intervals are reproduced in Figs. 34-43.

Motion photomicrography of this group of cells was begun 145 minutes after inoculation. The times given in Table IV are correct in a relative sense, but do not indicate the actual duration spans of all of the cells from times of fission. They are, however, relatively if not absolutely significant. After this group was placed under observation, fission occurred in 5 cells, producing progeny approximately one-half the size of the parents. It is estimated that these cells were

TABLE IV—*Concluded*

Cell No.	Time after inoculation	Duration under observation	Length	Breadth	Volume	Volume increase	
						cubic micra	per cent
G1	min.	min.	micra	micra	cubic micra		
	481	492	2.86	1.39	4.21	1.11	26.3
	525		3.52	1.39	5.32	0.80	19.0
	578		3.32	1.39	5.01	-0.78	-18.7
	637		3.59	1.19	3.43	—	—
H	637.3		—	—	—	—	—
	481	616	2.92	1.33	4.06	0	0
	750		2.93	1.33	4.06	—	—
	761		—	—	—	—	—
H1	481	633	3.99	1.33	5.51	—	—
	777		3.05	1.33	4.24	-1.27	-22.8
	778		—	—	—	—	—
J	481	646	3.35	1.33	4.61	0.1	2.1
	761		3.46	1.33	4.71	1.2	26.0
	762		3.46	1.46	5.81	-0.2	-4.3
	790		2.93	1.39	4.41	-0.91	-19.5
	791.6		2.67	1.33	3.7	—	—
	791.7		—	—	—	—	—
	481	550	3.46	1.33	4.8	-0.74	-15.4
K	694.7		2.93	1.33	4.06	-0.74	-15.4
	695.1		2.93	1.33	4.06	—	—
	695.3		—	—	—	—	—
	481	493	3.72	1.33	5.17	-1.43	-27.6
K1	638.2		3.33	1.19	3.74	—	—
	638.3		—	—	—	—	—
	481	617	4.12	1.19	4.63	0	0
M	461.6		4.12	—	—	—	—
	761.1		—	—	—	—	—
	481	566	3.86	1.39	5.81	1.22	21.0
	495		3.99	1.39	6.21	1.40	24.1
N	525		4.12	1.39	6.03	-0.40	-6.8
	710.9		3.59	1.39	5.41	-0.83	-14.2
	711.1		3.59	1.33	4.93	—	—
	711.4		—	—	—	—	—
	481	507	3.06	1.33	4.25	0	0
O	642		3.06	1.33	4.25	-1.04	-24.5
	650		2.93	1.19	3.29	—	—
	652		—	—	—	—	—
	481	497	2.67	1.33	0	0	0
O1	637		2.67	—	—	—	—
	642		—	—	—	—	—

Most of the 18 cells which underwent lysis were from 1.5-5 times as large as cells in the normal cultures at ages of 10-14 hours. This abnormal bulk might be taken to indicate swelling due to the action of the bacteriophage. The histories of the cells, however, indicate that a different interpretation is nearer to the truth. These cells are to be compared with cells of the approximate same generations, namely the third and fourth. It can be seen from Table IV and Text-fig. 3 that the cells undergoing lysis were never larger and were sometimes smaller than cells of the same generations. Occasionally the calculations indicated a slight increase in volume, but this amount was never greater than the experimental error in the measurement of the width of the cell. The measurements showed that the cells attained a certain size, within the normal range of sizes of cells of *B. megatherium*, and that no enlargement by growth or swelling preceded the ultimate lysis.

The terminal stage of lysis of *B. megatherium* was also a relatively slow process, slow enough to be followed by the eye. The cells became progressively more translucent, gradually lost definite outlines and finally disintegrated into a mass of granules and amorphous debris. The interior of the cell seemed to become less dense before the periphery lost its definite edge. This may have been an optical effect and not the indication of the persistence of a cell membrane, although the persistence of a cellular envelope is definitely suggested in some of the pictures. This terminal phase of lysis occupied 2-5 minutes. The beginning of the last stage is usually indicated in Table IV by decreases in volume values. We doubt whether an actual shrinkage occurred. The apparent decrease in volume is due probably to decrease in values of measurements of dimensions as a consequence of decreased optical density of boundary surfaces. It was hard to distinguish the outlines of the cells at this stage. There was certainly no evidence obtainable by measurement of unstained cells that the cells of *B. megatherium* enlarged or swelled in the final minutes of lysis. There was no evidence of an explosive bursting. The photographs show that the cells gradually disintegrated, leaving their residues within the areas previously occupied by the cells, as was clearly described by Cowles (10). The residue remaining after the lysis of *B. megatherium* was volumi-

without any progression of morphological changes suggesting the development of new forms. Our observations did not provide any evidence in favor of the theory that bacteriophagic lysis is an expression of a phase in the life cycles of these organisms.

Among the theories of lysis by autolysis and hydrolysis, Bronfenbrenner's (1, 11) is of particular interest. Hetler and Bronfenbrenner (11) have supplied chemical evidence that *Bact. coli* and other organisms undergo hydrolysis perhaps by the bacteriophagic activation of an endocellular enzyme. Bronfenbrenner's hypothesis, based on this and upon observations of cellular changes in moist and relatively dry media, is that products of hydrolysis increase the osmotic pressure inside the cell, causing water to enter, distend and eventually burst the cell. We do not agree with these investigators that their data give unequivocal evidence of hydrolysis of bacterial protein, and the time when the samples were taken for analysis indicates that the data obtained cannot be used to describe the state of the cell at the time of lysis. On general grounds also the notably particular reactions and resistance of bacterial cells to changes in osmotic pressure (Falk (12), Gotschlich (13), Raichel (14)) seem to us to offer opposition to a theory of bursting by osmotic pressure. The antigenic properties of the lysate seem to us to contradict the notion that extensive hydrolysis of bacterial protein is brought about by bacteriophage. Our observations are opposed to this explanation of lysis in their showing that swelling is not an invariable antecedent to lysis of *Bact. coli* and does not occur in *B. megatherium*, and that the residue remaining after lysis is in the form of particles and small blocks.

Our observations suggest to us that reduction of surface tension at the cell-medium interface and at the interfaces of particles of constituents within the cells may be an important factor in the mechanism of lysis. Frobisher (15) has described somewhat similar changes of size and shape of bacteria in solutions of soap, bile and bile salts having surface tension values of 35-40 dynes. Reduction of surface tension appeared to cause granulation and various degrees of swelling and dispersion of the cell substance by emulsification. Frobisher suggests that an additive factor may be the weakening of the retaining pressure that depends on the surface tension of the fluid surrounding the cell allowing any expansive property of the cell to cause it to burst. We

4. Bacteriophage spreads by contact through a group of cells and also along paths determined by genetical relationships.
5. A large amount of cellular debris remains after the lysis of the cells in both of these species of bacteria. This residue of material is in the form of irregularly shaped masses and granules. This material is not in solution at the time of lysis and appears not to be digested or hydrolyzed.
6. Theories of the mechanism of lysis are discussed. It is suggested that reduction of surface tension of the cells may be an important factor in the mechanism of lysis.

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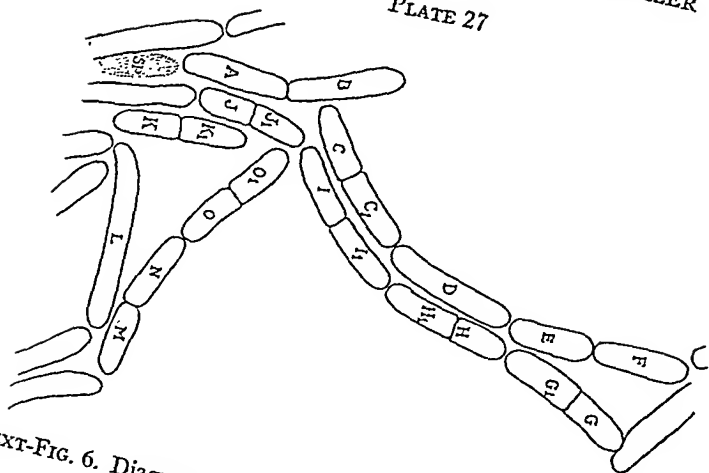
possible to renew the study of the problem and its relation to clinical disorders.

Method

The adult male dog was used in the present study. It was chosen because in this form accessory suprarenal cortical tissue is rarely found (2). On the other hand, accessory chromaffin tissue occurs in considerable amounts, especially in the abdominal chromaffin body, so that it is unlikely that a critical deficiency may occur, except in so far as proper function of the medulla may depend upon the integrity of the cortex (3). Lastly, the dog lends itself well to metabolism experiments involving repeated collections of urine and blood, and can be trained for studies of the respiratory metabolism. The operation of suprarenalectomy in the dog is more difficult than in some other forms, but with practice can be performed rapidly with little danger. The anesthetic was found to be of importance. The animals received a preliminary injection of morphine, and care was taken to avoid excessive or prolonged etherization. They recovered promptly and generally in an hour or two were standing up in their cages, eating on the following day. A loss of 0.2 to 0.5 kilos in weight usually occurred but was regained within a week. The animals have been suprarenalectomized in two stages, the intervals between operations varying from 1 to 5 weeks. Most of the operations (Swingle, Firor, Penick, Weinstein) were done from the lumbar approach, but several were also performed by the abdominal route (Widenhorn). In all, a group of about sixty animals have been studied during the past 2 years, and form the basis of this report. The possible effects of estrus or of pregnancy have been avoided by the exclusive use of male animals. In every instance the gland was removed intact, without tearing, in the surrounding fatty connective tissue. From this it was carefully dissected out, weighed, and the medulla was then removed by dissection underneath a hand lens for chemical analyses. We cannot assert that accessory cortical tissue does not exist. We can say that careful search at autopsy has uniformly failed to reveal its presence, that withdrawal of injections of the cortical extract has always been followed by the characteristic symptoms of insufficiency, and that in every instance the animal has died when injections were not resumed in time to prevent such an outcome.

The animals have been kept in well ventilated quarters in individual cages. They have been maintained on a standardized diet with daily exercise in the open air. Vermifuges were administered when indicated prior to operation. Further details as to care of the animals and the administration and assay of the cortical extract have been given in a recent article on a biological method for assay of the hormone (4).¹ All of our dogs are now vaccinated as routine against distemper before operation.

¹ The dog unit is defined as the minimum daily kilo dose of cortical hormone necessary to maintain normal physiological conditions in the suprarenalectomized,



TEXT-FIG. 6. Diagram of cells of *B. megatherium* shown in Figs. 34-43.

FIGS. 34-43. Serial photographs of morphological changes in cells of *B. megatherium* in culture on agar medium undergoing lysis by bacteriophage. Enlargements from motion picture film made Jan. 5, 1932. Exposures at the rate of 2 per minute during first 9 hours of growth and at 30 per minute during the next 6 hours. $\times 2000$. For diagram of cells see Text-fig. 6.

Times after inoculation are as follows: Fig. 1, 564 min.; Fig. 2, 565 min.; Fig. 3, 566 min.; Fig. 4, 620 min.; Fig. 5, 637 min.; Fig. 6, 642 min.; Fig. 7, 694 min.; Fig. 8, 762 min.; Fig. 9, 778 min.; Fig. 10, 860 min.

The pictures show the slow disintegration of the cells. The cell image gradually fades out during 2 to 10 minutes. A granular debris remains in the place formerly occupied by the cells.

noticeable immediate physiological effect. No symptoms of overdosage were detectable in the intact animal even after continued

TABLE I

Effect of Cortical Extract Injection on Blood of the Intact Animal. No Evidence of Discomfort or Abnormality of Behavior after the Injection

Dog 8, weight 14.4 kilos.

Date	Non-protein nitrogen	Sugar	Creatinine	Serum phosphate	Serum cholesterol	Plasma proteins	Albumin Globulin ratio	Oxygen capacity
1930	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	gm. per 100 cc.		vol. per cent
Oct. 8								
9.00 a.m.	30	100	1.3	2.7	130	6.3		
1.00 p.m.	26	95	1.4	3.3	111	5.2		18.1
25 cc. extract intravenously (approximately 150 dog units)								
2.00 p.m.	22	95	1.3	3.3	120		34/66	17.7
3.00 p.m.	26	97	1.3	3.4	111	5.4	41/59	17.8
4.00 p.m.	24	95	1.3	3.4	103	5.3		17.6
5.00 p.m.	21	90	1.3	3.9	—	5.5		16.9
Oct. 9								
9.00 a.m.	22	75	1.3	2.7	83	5.5	42/58	17.8

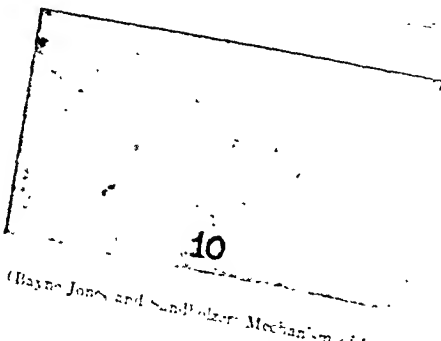
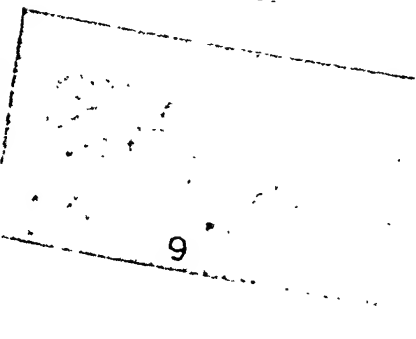
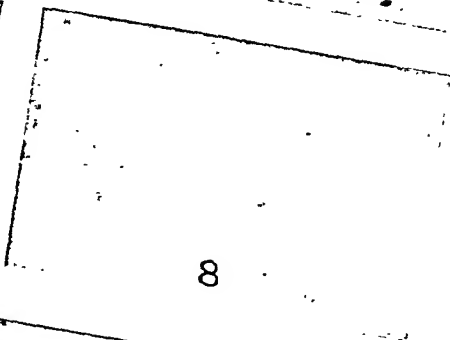
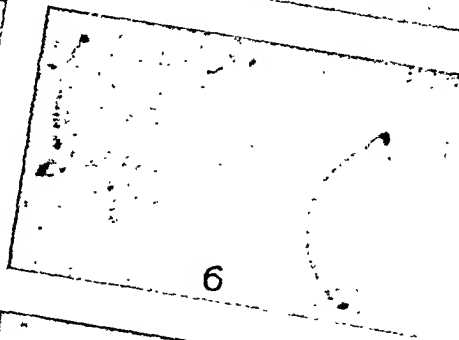
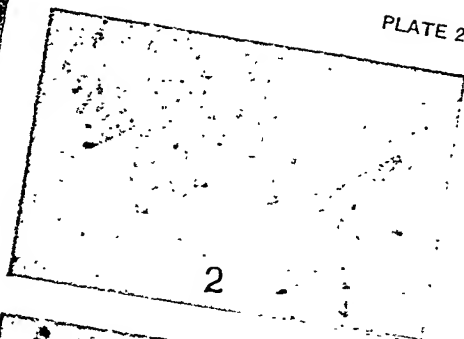
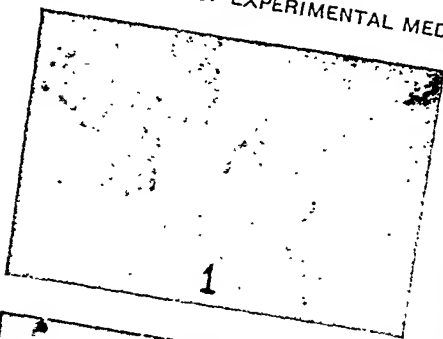
TABLE II

Effect of Continued Daily Injections of Cortical Extract on Blood of the Normal (Intact) Animal. No Change in Weight or Nutrition

Dog 8, weight 14.4 kilos.

Date	Non-protein nitrogen	Creatinine nitrogen	Sugar	Serum inorganic phosphates	Plasma cholesterol	Plasma proteins
1930	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	gm. per 100 cc.
Oct. 9	22	1.3	75	2.7	83	5.5
Thereafter injections of 5 cc. cortical extract (40 dog units) daily						
Oct. 16	29	1.5	85	4.1	166	6.0
" 22	33	1.4	66	3.3	166	5.3
" 28	30	1.3	72	3.0	120	5.6
Nov. 5	26	1.5	75	3.7	150	5.5

injections of large doses over a period of a month (Table II). This hormone accordingly resembles, in its absence of toxic effect, that of gonadal or anterior lobe extracts, and the findings are in con-

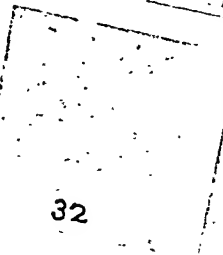
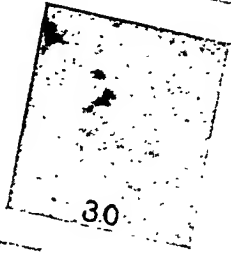
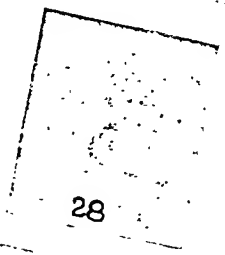
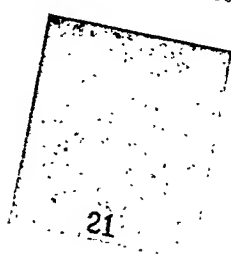
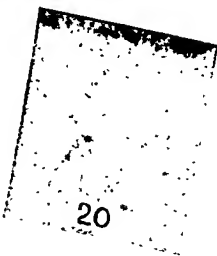
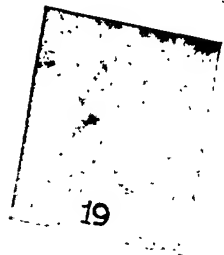
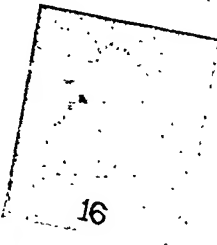
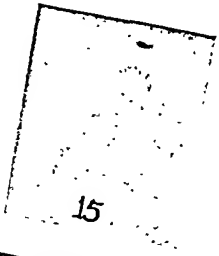
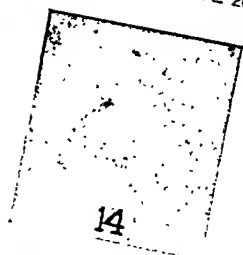
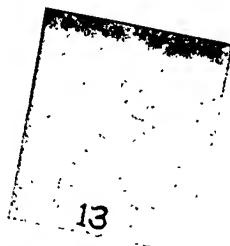
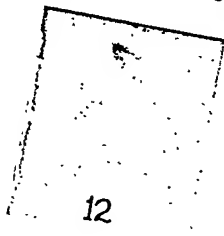


trast to the toxic effects which result promptly from overdosage with insulin, adrenalin, and the extracts of the thyroid and parathyroid glands. No change which is constant or significant in character has been detected in any of the blood constituents studied. We do not believe such changes can be made the basis of a method for assaying the cortical extract, in concentrations, at least, as at present employed.

(b) Maintenance of Suprarenalectomized Dogs with Cortical Extract

The bilaterally suprarenalectomized animals have been treated with subcutaneous injections of cortical hormone twice daily, using the customary maintenance dose as above determined. During the first few days after operation, and until the appetite is well reestablished, and superficial wound infections, if present, healed, we give larger doses. Under such circumstances the animals are active, maintain their weight, take food eagerly, and exhibit the sexual interest of normal dogs. Blood pressure, pulse, and body temperature are kept at normal levels. The subcutaneous fat is not diminished and resistance to infection is well maintained. The hair grows back quickly and profusely at the site of operation. There are no characteristic changes in the blood constituents studied. Our experiments indicate that the maintenance dosage is higher in the growing animal.

Dogs suffering from wound infections, intestinal parasites, or subjected to severe surgical trauma, or which are permitted to go into advanced insufficiency before resuscitation with extract may readily lose 20 to 25 per cent of their body weight and require a prolonged period of nursing with the administration of large amounts of the extract before they gain weight and return to a normal condition. Withdrawal of considerable amounts of blood at any one time, or repeated small blood withdrawals from these animals have a decidedly unfavorable effect. Chronically undernourished, underweight animals, even if the inanition is not due to deep wound infection or to worm infestation, usually have a higher extract requirement. Animals which are not permitted to lapse into severe insufficiency, and upon which exacting experimental procedures or blood withdrawal are only infrequently performed remain in good condition. While not unduly sensitive to cold, suprarenalectomized animals on daily extract injections do badly if subjected to extremes of temperature. Operations



BLOOD NON-PROTEIN NITROGEN
BLOOD UREA NITROGEN
URINE NITROGEN } IN ADRENAL CORTICAL INSUFFICIENCY

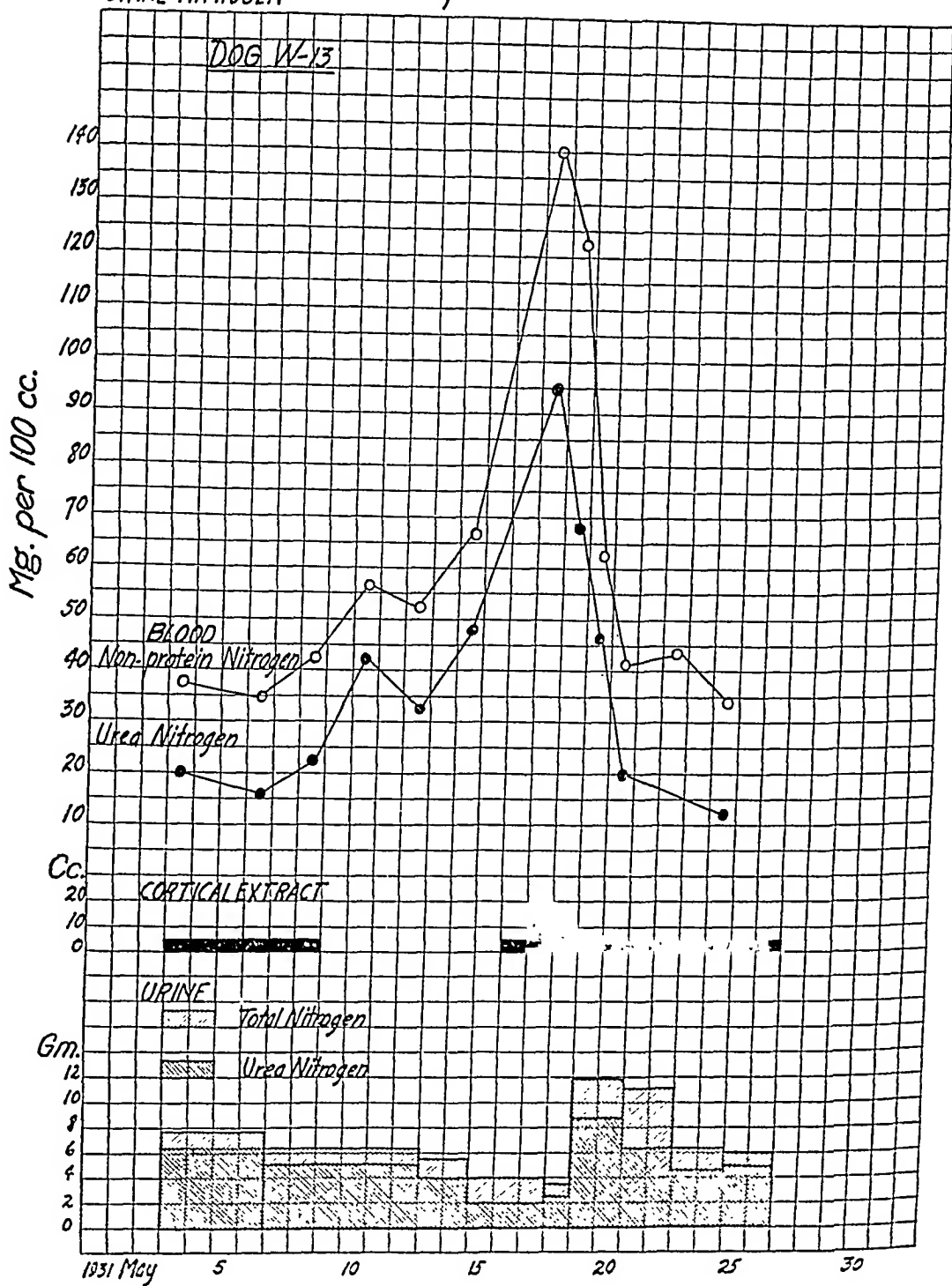
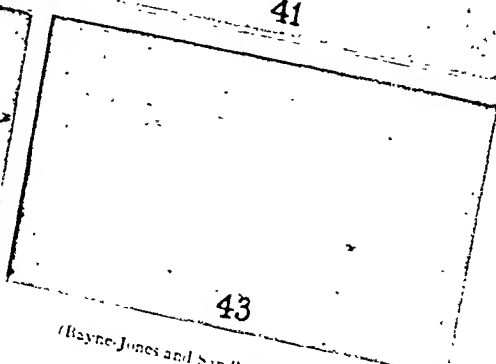
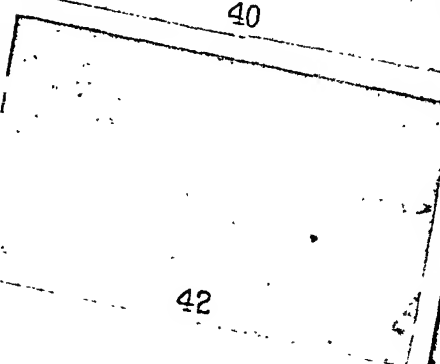
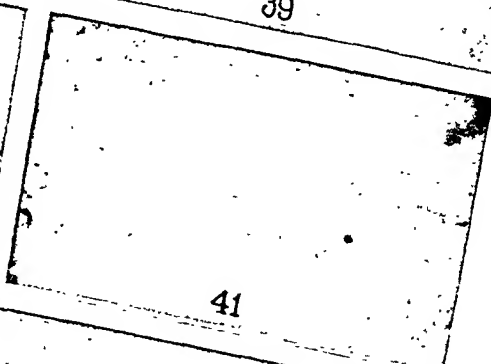
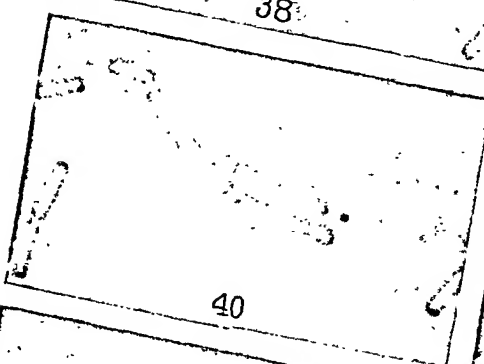
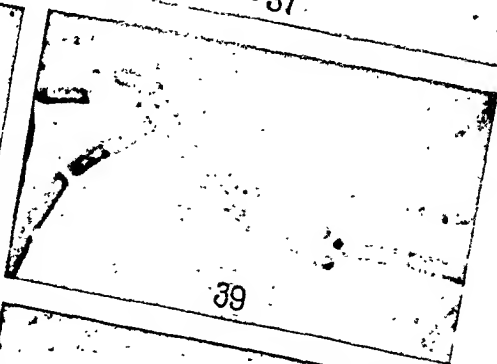
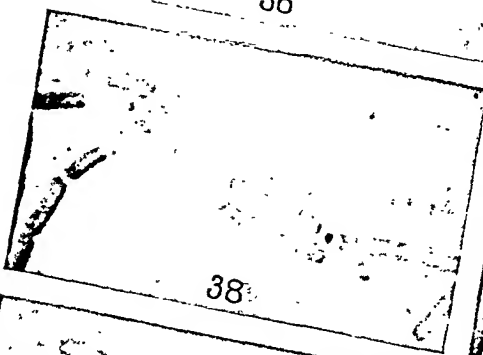
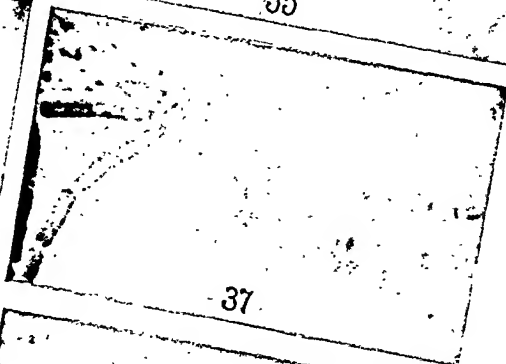
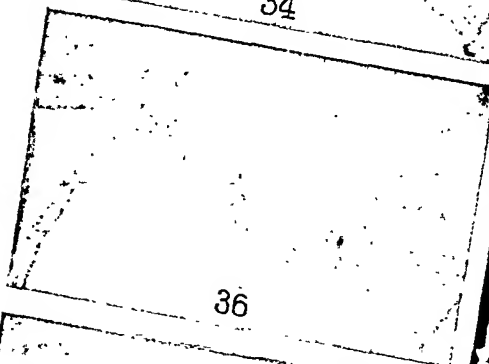
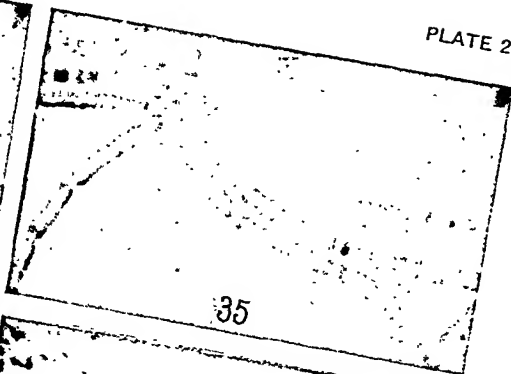
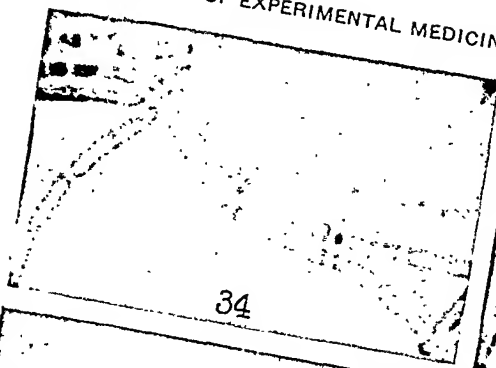


CHART 1



BLOOD NON-PROTEIN NITROGEN }
 BLOOD UREA NITROGEN } IN ADRENAL CORTICAL INSUFFICIENCY
 URINE NITROGEN }

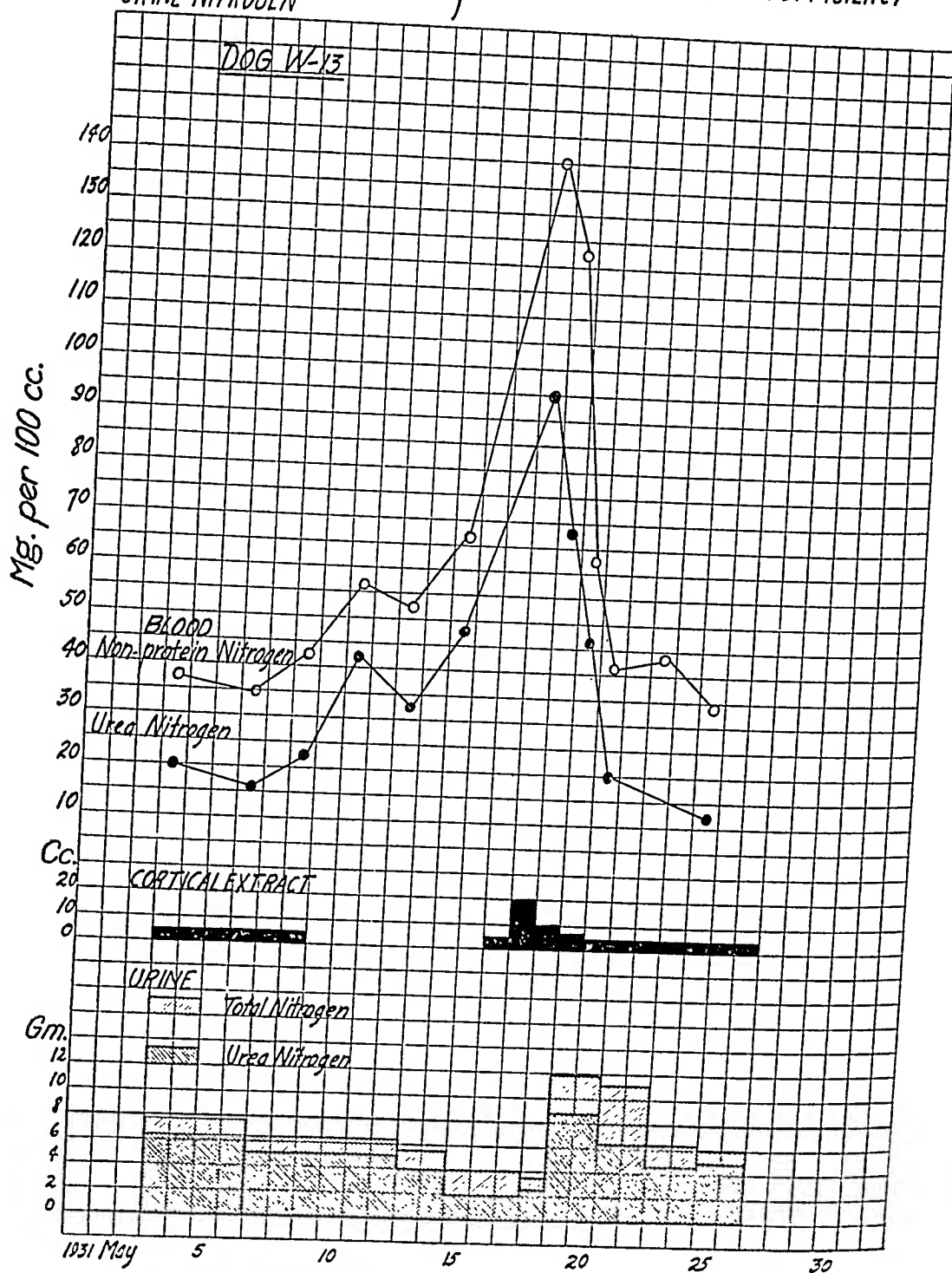


CHART 1

STUDIES ON THE SUPRARENAL CORTEX

I. CORTICAL SUPRARENAL INSUFFICIENCY AND THE ACTION OF THE CORTICAL HORMONE UPON THE NORMAL AND SUPRARENALECTOMIZED DOG*

By GEORGE A. HARROP, JR., M.D., AND ALBERT WEINSTEIN,† M.D.
(From the Chemical Division of the Medical Clinic, Johns Hopkins University and Hospital, Baltimore)

(Received for publication, September 27, 1932)

Investigation of the physiological function of the suprarenal glands has presented many difficulties. The intimate relation of the cortex to the medullary portion of the gland, which contains adrenalin (or its precursor), has rendered it hard to separate the cortical material from the latter substance. Furthermore, the fatal outcome of removal of the glands (in the absence of accessory cortical tissue), within a short time after operation tends to confuse the results of the surgical procedure with those due solely to ablation of the cortex. The introduction of an extract of beef suprarenal cortical tissue (1), nearly free of adrenalin and of lipids, the parenteral use of which prolongs indefinitely the life of suprarenalectomized cats, now makes it

* Dr. W. M. Firor has done many of the operations for suprarenalectomy and has developed important details in the operative technique. Several animals were prepared at Princeton and shipped to Baltimore for the experimental work by Dr. Swingle. The operations during the early stages of the work were done with the collaboration of Dr. Hans Widenhorn. Dr. R. Penick has done several months of the past spring. The remainder of the operations, and all during recent months have been done by Weinstein and Harrop. The cortical extract used was furnished in part from the Department of Biology, Princeton University, and in part was prepared in the Chemical Laboratory of the Medical Department, Johns Hopkins University and Hospital. Preliminary reports of various portions of this work were published in *Science*, 1931, 73, 683; *Tr. Assn. Am. Physn.*, 1931, 46, 138; 1932, 7, 274; *Transactions of the American Society for Clinical Investigation*, *J. Clin. Phys.*, 1932, 11, 816.

† John D. Archbold Fellow in Medicine.

During the last few days of November 1/3 cc. of extract per kilo per day proved to be inadequate, the dog's weight dropped to 12.6 kilos, the food intake was low, and the non-protein nitrogen was 62 mg. per 100 cc. A larger dosage was temporarily required.

In December the animal copulated with a bitch in heat. The non-protein nitrogen rose to 66 mg. per 100 cc. The dog looked ill during the following week and in spite of 0.8 to 1.2 cc. of extract per kilo per day, the appetite remained poor and the weight fell from 12.6 to 11.3 kilos.

During the latter part of December a skin infection developed while the dog was on 0.5 cc. of extract per kilo, the non-protein nitrogen rose to 70 mg. per 100 cc., and there was clear evidence of insufficiency.

In Jan., 1932, 11 months after the second stage operation the animal was in fair condition but the weight was 12.0 kilos. The extract requirement was now found to be about 0.7 cc. per kilo, per day, as compared to 0.25 to 0.3 cc. per kilo, which was the maintenance dose found adequate during the early weeks after the removal of the suprarenal glands. A metabolism experiment was begun Jan. 4, 1932, in which a measured fluid intake was forced by mouth. 2 days after the withdrawal of extract, Jan. 11, the blood non-protein nitrogen rose from 50 to 92 mg. per 100 cc. and on the 6th day, with marked evidences of insufficiency, it had reached 145 mg. per 100 cc. Weight 11.3 kilos. The extract was resumed in large amounts, 5.5 cc. per kilo and the next morning the non-protein nitrogen was 88 mg. per 100 cc. 2 cc. of extract per kilo was then given, and 24 hours later the blood non-protein nitrogen was 56 mg. per 100 cc. The following day 1.9 cc. of extract per kilo was given, and after a further 24 hours the blood non-protein nitrogen was 35 mg. per 100 cc. and the dog was active, eating, and well. Its weight did not increase.

Jan. 26, weight 10.7 kilos. The dog was placed on an extract rendered inactive by accidental overheating, which was administered in large amounts (1 to 2 cc. per kilo of weight). During the following 2 days it ate very little and the non-protein nitrogen rose to 80 mg. per 100 cc. The weight dropped to 10.3 kilos. The animal was found dead the following morning, Jan. 29, 11 months and 27 days after removal of the second suprarenal gland.

Autopsy by Dr. Kindell revealed no evidence of infection. A peptic ulcer was present. There was no lymphoid or thymic hyperplasia and no explanation for death could be found. The suprarenal glands had been completely removed and no accessory tissue, cortical or medullary, was found after careful search. Examination of the other glands of internal secretion revealed no significant changes. The thyroid gland showed no evidence of hyperplasia; there was some calcification of the colloid material. All of the internal organs showed atrophic changes, associated with the undernutrition. This was especially marked in the liver which showed central atrophy. There were areas of necrosis in the spleen, which may possibly have been associated with toxic changes due to the use of the batch of extract given Oct. 27 to 29 which had been prepared in another laboratory and which produced death in one other suprarenalectomized animal, and in a normal

The following studies are reported in the present communication: (a) the effect of injection of the cortical extract into normal animals; (b) the maintenance of suprarenalectomized animals with cortical extract; (c) the clinical picture of suprarenal insufficiency, due to extract withdrawal, and the subsequent recovery following resumption of injections; finally (d) a search was made for possible changes in various blood constituents in the suprarenalectomized animal during the various phases, including a reexamination of the evidence for blood concentration.

(a) *Effect of the Cortical Hormone upon Normal Animals*

Because of the current confusion as to the results of injections of the cortical hormone into normal animals we present typical protocols showing the effects of such injections upon certain blood constituents. (Tables I to IV). Amounts of the material up to 100 cc. in bulk (Table III), and assaying up to approximately 1800 dog units in strength (Table IV), were given in a single intravenous injection without any

adult, well nourished, male dog for a period of 7 to 10 days; the two criteria of normal physiological condition being maintenance of body weight and constant level of blood non-protein nitrogen (or urea). According to this definition the hormone content of different batches of our stock cortical extract varies from 4 to 10 dog units per cc. The assay value of the new whole gland extracts (5) vary from 40 to 100 dog units per cc. We have been using the whole gland extracts since Mar., 1932.

Adult dogs are selected, free of infection and worm infestation. They are suprarenalectomized in two stages, and the success of the operation is established after complete healing of the wounds by production of symptoms of insufficiency by withdrawal of extract. The dogs are then placed in separate cages and given 0.5 cc. extract per kilo subcutaneously in two equally divided doses daily, morning and evening, until it is demonstrated that the blood non-protein nitrogen (or urea) is at a normal and constant level, that the weight is maintained, the food properly eaten, and the clinical condition in every respect normal. The ration given is constant and consists of 80 calories per kilo per day of bal-ra dog ration with 1 cc. cod liver oil three times weekly and 1 gm. vitavose per kilo three times weekly on alternate days. The dogs are permitted to run in the open air, weather permitting, at least 2 hours daily. Certain animals refuse to take the ration indicated above and fresh beef, milk, and horse meat have been used in such cases for temporary or extended periods in order to produce a proper state of nutrition. Suprarenalectomized dogs require meticulous care in all details of their management.

animal was subjected repeatedly to exacting experimental procedures, and was in a state of marked insufficiency on at least seven different occasions and in mild insufficiency (anorexia, rise in blood non-protein nitrogen, loss of weight) on several other occasions. The maintenance requirement of extract seemed to bear a relation to the state of nutrition. Different batches of extract were used, but the assay value as tested on other animals did not vary greatly. The time required for the development of insufficiency after discontinuance of extract (Table V) bears a rather definite relation to the state of nutrition, as indicated by the body weight. The only exceptions to this rule are the fifth and sixth periods of insufficiency during the summer of 1931, in warm weather, which has been stated to shorten survival periods of suprarenalectomized animals (7).

It is evident from the extended study of this dog and of our other animals, that renal insufficiency characterized by inadequate secretion of urine and of certain urinary elements during the acute stage of suprarenal insufficiency (6), does not persist after recovery is brought about by the use of large amounts of cortical extract; nor are anatomical lesions, indicating renal damage, to be found at autopsy in animals which have been repeatedly in insufficiency.

The experiment on this animal, confirmed on several others, also indicates the failure of our efforts to maintain bilaterally suprarenalectomized dogs on feedings of freshly dissected ground beef cortex alone without the aid of extract.

(c) Clinical Picture of Suprarenal Insufficiency Following Extract Withdrawal

Following the withdrawal of daily injections of cortical extract from suprarenalectomized dogs with completely healed operative wounds, a period of 2 to 10 days ensues during which no abnormality is observed. The earliest sign of insufficiency is anorexia, and generally a lessened fluid intake. This usually occurs abruptly. When the food intake on the standardized diet is weighed each day, the drop in consumption is very striking, and it produces a prompt loss of body weight. The time of onset of anorexia varies with the individual animal and especially with the state of nutrition. Frequently the animal may be tempted with fresh meat after the previous ration is refused, but

TABLE III
Effect of Cortical Extract Injection on Blood of the Intact Animal. No Evidence of Discomfort or Abnormality of Behavior after the Injection
Dog 4, weight 14.5 kilos.

Date	Non-protein nitrogen	Sugar	Creatinine	Serum phosphate	Plasma cholesterol	Plasma proteins	Oxygen capacity
	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	gm. per 100 cc.	vol. per cent
1930							
Oct. 10							
9.00 a.m.	40	85	1.4	4.1	78	4.8	16.4
1.00 p.m.	35	90	1.4	3.4	82	4.9	
1.30 p.m.	100 cc. extract intravenously (approximately 600 dog units)						
1.40 p.m.	36	90	1.4	4.1	81	4.5	16.5
2.30 p.m.	36	95	1.4	4.1	83	4.4	15.5
3.30 p.m.	33	92	1.4	3.9	76	4.8	15.1
4.30 p.m.	38	95	1.4	4.1	50	4.4	15.0
9.30 p.m.	32	95	1.4	4.3	57	4.1	15.1
Oct. 11							
9.00 a.m.	33	95	1.4	3.3	53	4.5	15.3

TABLE IV
Effect of Cortical Extract Injection on Blood of Intact Fasting Animal (Animal Fasted Commencing 15 Hours before and throughout Course of the Experiment)
Dog F-39, weight 10 kilos.

Date	Non-protein nitrogen	Sugar	Plasma chlorides	Plasma cholesterol	Red blood cells	Hemoglobin	Volume of packed red cells (hematocrit)	Mean corpuscular volume	Mean corpuscular hemoglobin	Mean corpuscular hemoglobin concentration
	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	$\times 10^4$	gm.	cc.	c. μ		per cent
1932										
May 31										
10.00 a.m.	34	82			6.90	15.8	54.0	77		
June 1										
10.00 a.m.	26	98	488	90.9	7.80	15.1	52.4	67	19	27
10.30 a.m.	30 cc. cortical Extract H63-66 intravenously (approximately 1800 dog units)									
11.30 a.m.	30	100	484	90.9	7.65	14.0	49.0	64	18	29
2.30 p.m.	24	98	496	90.9	7.38	15.0	53.6	73	20	28
June 2										
10.00 a.m.	30	82	496	90.9	7.38	15.0	53.6	73	20	28

Extract H 63-66 had a strength by dog assay of 60 units. The erythrocyte and hemoglobin measurements were made by Dr. M. Wintrobe.

animal was subjected repeatedly to exacting experimental procedures, and was in a state of marked insufficiency on at least seven different occasions and in mild insufficiency (anorexia, rise in blood non-protein nitrogen, loss of weight) on several other occasions. The maintenance requirement of extract seemed to bear a relation to the state of nutrition. Different batches of extract were used, but the assay value as tested on other animals did not vary greatly. The time required for the development of insufficiency after discontinuance of extract (Table V) bears a rather definite relation to the state of nutrition, as indicated by the body weight. The only exceptions to this rule are the fifth and sixth periods of insufficiency during the summer of 1931, in warm weather, which has been stated to shorten survival periods of suprarenalectomized animals (7).

It is evident from the extended study of this dog and of our other animals, that renal insufficiency characterized by inadequate secretion of urine and of certain urinary elements during the acute stage of suprarenal insufficiency (6), does not persist after recovery is brought about by the use of large amounts of cortical extract; nor are anatomical lesions, indicating renal damage, to be found at autopsy in animals which have been repeatedly in insufficiency.

The experiment on this animal, confirmed on several others, also indicates the failure of our efforts to maintain bilaterally suprarenalectomized dogs on feedings of freshly dissected ground beef cortex alone without the aid of extract.

(c) Clinical Picture of Suprarenal Insufficiency Following Extract Withdrawal

Following the withdrawal of daily injections of cortical extract from suprarenalectomized dogs with completely healed operative wounds, a period of 2 to 10 days ensues during which no abnormality is observed. The earliest sign of insufficiency is anorexia, and generally a lessened fluid intake. This usually occurs abruptly. When the food intake on the standardized diet is weighed each day, the drop in consumption is very striking, and it produces a prompt loss of body weight. The time of onset of anorexia varies with the individual animal and especially with the state of nutrition. Frequently the animal may be tempted with fresh meat after the previous ration is refused, but

are badly tolerated in hot weather and recovery even with adequate extract is more difficult to bring about. Longhaired dogs seem to be more resistant to the effects of suprarenalectomy, possibly because they are better insulated to temperature changes. Not infrequently an animal subjected to experimentation will sink gradually into a condition of chronic inanition and finally die in insufficiency, autopsy showing no unusual findings or infection. This may occur, in spite of careful nursing, feeding, and apparently adequate amounts of extract. Feeding of fresh beef suprarenal cortical tissue, milk, 20 per cent cream, yeast concentrates, and cod liver oil, as well as oil injections (yanol) intramuscularly, appear to benefit such animals; but the results are not always successful.

We have now had a large group of animals bilaterally suprarenalectomized, with survival periods varying between 2 months and 1 year. We present the protocol of an animal which survived bilateral suprarenalectomy for 360 days, death finally resulting from a relapse due to an emergency in which we lacked actively potent extract for experimental use. We are not yet certain that injections of the cortical extract will maintain the suprarenalectomized animal in normal condition, provided the nutrition is properly preserved, over indefinite periods, but there is at the present time no evidence to the contrary.

Protocol of Clinical Course of Dog W-13.—A male collie, weight 13.2 kilos, was suprarenalectomized in two stages (Widenhorn). The left suprarenal gland was removed Nov. 21, 1930, and the right one Feb. 2, 1931, both by the abdominal route. The dog died 11 months and 27 days later in suprarenal insufficiency. The original operative wounds healed well. Cortical extract was withheld to test the possible existence of accessory cortical tissue. 8 days later, Feb. 10, the blood non-protein nitrogen was 104 mg. per 100 cc., weight 12.3 kilos, the dog showed complete anorexia, was apathetic, and had diarrhea. 1 cc. of cortical extract per kilo of body weight was given, and the next morning the dog was active and normal in all respects. The blood non-protein nitrogen had dropped to 57 mg. per 100 cc.

An assay of a lot of extract was then made, and of the material used 1/6 cc. per kilo was adequate. On 1/8 cc. per kilo, in 5 days (Feb. 26), the non-protein nitrogen rose to 84 mg. per 100 cc., the dog refused food and looked sick. He was given a larger dose of extract and recovered. In Mar., 1931, the animal was placed in a metabolism cage to study the effects of gradual withdrawal of extract. During this experiment, after the dog had been on 1/6 cc. per kilo of extract for 3 days, the non-protein nitrogen rose to 82 mg.

its former smoothness. The relative ineffectiveness of the extract in the presence of infection is just as striking as is that of insulin under similar conditions. Deep chronic infections at the site of operative wounds, detected only at autopsy, have on a number of occasions confused us before death of the animal, sometimes weeks after operation, because of the apparent lack of response even to large amounts of potent material.

The effect of injections of the cortical hormone, where the insufficiency is well established, is not immediate in the dog, and objective improvement cannot usually be demonstrated for a period of 12 to 24 hours after a large resuscitating dose is given. It is practically impossible to induce recovery of animals which are almost moribund when injections are commenced. The slower reaction of the prostrate dog to the action of the hormone contrasts with the effect which has been reported for other species of animals. The amount of extract required to bring the animal out of insufficiency is invariably much greater than the maintenance requirement. In this it resembles thyroid insufficiency (*e.g.*, myxedema).

While the typical symptoms of suprarenal insufficiency, as described above, occur in most dogs, a small group, when deprived of extract, after the usual fore period without symptoms, will die in a relatively brief time and with little warning. These are usually dogs markedly underweight and not in prime condition. Such animals may be seen late in the evening, apparently alert and in fair condition, with normal temperature and pulse, and with normal gait, and may then be found dead next morning. In such cases the blood non-protein nitrogen may not be elevated, as discussed below, within 6 or 8 hours of death; and even actually at death we have taken blood from two dogs which showed values not greatly out of the normal range for non-protein nitrogen, sugar, chlorides, and cholesterol. It is fair to say, however, that in no fatal case have we failed to see a marked grade of anorexia for at least 24 to 48 hours before death, and this we always regard as a significant symptom. The autopsy findings in such animals differed in no essential detail from the pathological changes observed in the other animals. Exercise appears to aggravate the symptoms of insufficiency.

The causes of death have been postoperative infection, sometimes

per 100 cc. (Mar. 19) and the animal was in insufficiency. Weight 10.2 kilos. The dog was rescued again by the use of large amounts of cortical extract, and the weight quickly rose to 12.3 kilos.

During the next 2 months experimental procedures were withheld. During this time the blood non-protein nitrogen fluctuated between 40 and 50 mg. per 100 cc. The dog looked very well and the weight rose to 15.5 kilos.

During the middle of May another metabolism experiment was performed and on the 11th day following withdrawal of extract injections, May 18, the blood non-protein nitrogen was 140 mg. per 100 cc., and the dog showed typical evidences of insufficiency. Weight 14.7 kilos. On large amounts of extract the non-protein nitrogen fell, on successive days, to 122, 62, and then to 41 mg. per 100 cc. The appetite returned, but there was no increase in weight. The non-protein nitrogen finally reached 33 mg. per 100 cc. During this period of relapse there was no definite urinary retention, but a diuresis was associated with recovery.

No further experimental procedures were carried out during the following 10 weeks and the animal's condition remained excellent on a maintenance extract dosage. On July 16, the dog weighed 16 kilos. Extract was then withdrawn and it was fed 10 gm. of fresh hashed beef suprarenal cortex per day from which the medulla had been carefully dissected. After 4 days the dog appeared to be in mild relapse. Weight 15.5 kilos, the blood non-protein nitrogen was 60 mg. per 100 cc. The mouth feedings of cortex were stopped and injections of extract resumed.

Aug. 3, weight 15.2 kilos, injections of extract were stopped and the dog was fed daily 30 gm. of fresh beef adrenal cortex prepared as above. The blood non-protein nitrogen on the 3rd day had risen to 72 mg. per 100 cc., food was refused, and the weight dropped to 14.7 kilos. It was again necessary to discontinue the use of the cortex feedings. On Aug. 24, weight 15.0 kilos, the dog was deprived of extract for 4 days. The non-protein nitrogen rose to 92 mg. per 100 cc., there was anorexia, weight decreased to 14.4 kilos, and the usual evidences of insufficiency were observed. At this time, 7 months postoperative, it was necessary to give the dog 0.5 cc. of extract per kilo per day to keep it normal. The weather during this period was very warm.

Early in Sept., 1931, the dog looked well and the weight was 14.4 kilos. It was active in all respects. The dog was deprived of extract for 3 days (Sept. 19), when it refused food, the weight dropped to 14.1 kilos and the non-protein nitrogen rose from 36 to 90 mg. per 100 cc.

On Oct. 9, weight 14.3 kilos, an insulin injection, 2 units per kilo intravenously, caused the dog to have convulsions; and, even after the administration of sugar by mouth, intravenously, and subcutaneously, the blood sugar for 6 hours remained at the level of 20 to 30 mg. per 100 cc. A few days later, while the animal was being given an extract of low potency, the non-protein nitrogen rose to 172 mg. per 100 cc. and the dog showed clear evidences of insufficiency. During the last few days of October (Oct. 27 to 29) it was placed on a batch of extract furnished us for assay by a pharmaceutical house (which later proved to be without potency). After this material had been used for 2 days the non-protein nitrogen was 70 mg. per 100 cc. and the dog was ill. Potent material was then given.

showed a small subcutaneous abscess at the site of extract injection over the lateral abdominal wall, and general emaciation. The suprarenal glands were cleanly removed.

TABLE VII

Changes in Certain Constituents of the Blood (Femoral Artery Blood) Following Cortical Extract Withdrawal and during the Suprarenal Insufficiency So Produced

Dog 13, male collie. Nov. 21, 1930, right suprarenalectomy. Feb. 2, 1931, left suprarenalectomy (Dr. Widenhorn).

Date	Weight	Temperature	Pulse	Cortical extract	Food	Activity	Blood analysis			
							Non-protein nitrogen	Urea nitrogen	Creatine nitrogen	Sugar
1932	kg.	°F.		cc. per kg.			mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
Mar. 9	11.5	101.3	70	0.25	+++	Normal	44	22.5	1.4	85
" 10				0.25	+++	"				
" 11				0.25	+++	"				
" 12				0.25	+++	"				
" 13	11.1	101.4	64	0.18	+++	"	50	30.0	1.5	70
" 14				0.18	++	"				
" 15				0.18	+	Fair				
" 16	10.7	98.8	112	1.0	0	Swaying spastic gait	82	64.0	1.6	60
" 17	10.5	100.9	110	1.0	+	Gait still swaying	65	45.0	1.6	70
" 18	10.2	100.8	114	1.0	+	Fair	58	45.0	1.6	70
" 19				1.0	+	"				
" 20	10.2	100.8	104	1.0	++	Normal	45	26.0		
" 21	10.4			1.0	+++	"				
" 22				1.0	+++	"				
" 23	11.1	101.0	60	1.0	+++	"	45	23.0	1.5	75
" 30	11.4	101.0	76	1.0	+++	"	43	24.0	1.7	80

* This cortical extract assayed approximately 4 units per cc.

per 100 cc. (Mar. 19) and the animal was in insufficiency. Weight 10.2 kilos. The dog was rescued again by the use of large amounts of cortical extract, and the weight quickly rose to 12.3 kilos.

During the next 2 months experimental procedures were withheld. During this time the blood non-protein nitrogen fluctuated between 40 and 50 mg. per 100 cc. The dog looked very well and the weight rose to 15.5 kilos.

During the middle of May another metabolism experiment was performed and on the 11th day following withdrawal of extract injections, May 18, the blood non-protein nitrogen was 140 mg. per 100 cc., and the dog showed typical evidences of insufficiency. Weight 14.7 kilos. On large amounts of extract the non-protein nitrogen fell, on successive days, to 122, 62, and then to 41 mg. per 100 cc. The appetite returned, but there was no increase in weight. The non-protein nitrogen finally reached 33 mg. per 100 cc. During this period of relapse there was no definite urinary retention, but a diuresis was associated with recovery.

No further experimental procedures were carried out during the following 10 weeks and the animal's condition remained excellent on a maintenance extract dosage. On July 16, the dog weighed 16 kilos. Extract was then withdrawn and it was fed 10 gm. of fresh hashed beef suprarenal cortex per day from which the medulla had been carefully dissected. After 4 days the dog appeared to be in mild relapse. Weight 15.5 kilos, the blood non-protein nitrogen was 60 mg. per 100 cc. The mouth feedings of cortex were stopped and injections of extract resumed.

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Early in Sept., 1931, the dog looked well and the weight was 14.4 kilos. It was active in all respects. The dog was deprived of extract for 3 days (Sept. 19), when it refused food, the weight dropped to 14.1 kilos and the non-protein nitrogen rose from 36 to 90 mg. per 100 cc.

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TABLE VIII

Changes in Certain Constituents of the Blood (Femoral Artery Blood) Following Cortical Extract Withdrawal and during the Suprarenal Insufficiency So Produced

Dog P-2, male, mongrel terrier. Suprarenalectomized by Dr. Swingle at Princeton during Feb., 1931.

Date	Weight	Temperature	Pulse	Cortical extract	Food	Activity	Non-protein nitrogen	Urea	Sugar	Serum potassium	Lactic acid	Serum total base
	kg.	°F.		cc. per kg.*			mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	m.-eq. per liter
Apr. 7	11.0	100.5	76	1.0	Well taken	Normal and active	56	40	72	16	7.0	
" 8				1.0	"	"						
" 9				1.0	"	"						
" 10	10.8	100.6	70	1.0	"	"						
" 11				None	"	"	54	35	80	20	8.6	153.6
" 12				"								
" 13	10.6	101.4	72	"	Very little							
" 14		100.0	106	"	None	Swaying gait, skin inelastic	84	64	70	20	7.1	
" 15 a.m.	10.0	98.4		2.0	"	"—vomiting	140	128	62			
" 15 p.m.		97.8	108	4.0	150 cc. 5% glucose		170	150	75	30	9.7	
" 16	9.7	106		3.0	150 " 5% "	Drinks water freely and is voiding.	170	150	75	25		
" 17	9.7	99.2	108	2.0	Small amount 150 cc. 5% glucose	No stool	200	180	115			
" 18	9.1			2.5	None	Walks much better. Diuresis (500 cc. per 24 hrs.)	170					
" 19	9.0			3.0	"	Large voiding	170	135	90			
" 20	8.8	98.0		2.0	"							
" 21	8.6	98.4	108	1.3	"		204	180	87	42		142.2
		Death										

In this animal the injections of the hormone were resumed either too late or in insufficient quantity to prevent a fatal outcome. Autopsy showed complete removal of the suprarenal glands, no accessory cortical tissue, and no evidence of infection.
* This cortical extract assayed approximately 4 dog units per cc.

dog. Autopsy on all three animals showed similar necrotic areas in the spleen. No significant changes were found in the kidneys.

In summary this animal lived almost a year (360 days) after suprarenal glands were removed. There were no noticeable pigmentary changes at any time in the mucous membranes of the mouth or in the

TABLE V
Relation of the Time Interval before Suprarenal Insufficiency after Extract Withdrawal, to the State of the Animal's Nutrition as Indicated by Body Weight
Dog W-13.

Period of suprarenal insufficiency	Date extract withdrawal	Weight kg.	Interval before insufficiency days	Weight kg.	Blood non-protein nitrogen mg. per 100 cc.	Comment
1	1931					
2	Feb. 2	13.2	8	12.3	104	
3	" 21	11.6	5	11.5	84	
4	Mar. 16	10.5	3	10.2	82	
5	May 7	15.5	11	14.7	140	
6	Aug. 24	15.0	4	14.4	92	
	Sept. 19	14.4	3	14.1	90	Warm weather
7	1932					" "
8	Jan. 11	11.7	6	11.3		
	" 26	10.7	2	10.3	145	Fluids forced
					80	Died following day

TABLE VI
Average Blood Non-Protein Nitrogen Level Mg. per 100 Cc. at Which Various Symptoms of Suprarenal Insufficiency Occurred in Twenty Bilaterally Suprarenalectomized Dogs

Average normal level	Anorexia and loss of weight	Gait changes	Vomiting	Diarrhea
mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
40	64	86	116	123

skin. The blood pressure was normal except during marked insufficiency. Kidney function (phenolsulfonephthalein excretion and creatinine and urea clearance) was impaired only when the degree of insufficiency had become marked. Albumin was never present in the urine and there were no changes in the urinary sediment. The

acidosis such as is found in the terminal stages of renal insufficiency may occur must remain open, but we do not believe it is a primary or serious factor in the production of suprarenal insufficiency.

In severe insufficiency blood samples are obtained with great difficulty; the veins are collapsed, the blood pressure is very low, and the blood is dark and thick. We do not doubt that the arterial and venous blood oxygen content difference is large; and this combined with the low oxygen consumption points to an extreme reduction, under these circumstances, in the circulatory minute volume. The venous blood from the jugular vein, which drains the cranial cavity, appears to be equally as dark as that from the femoral veins draining the muscle areas, and in the few instances studied the oxygen unsaturation was the same.

Blood Sugar.—The effect of suprarenal insufficiency upon the blood sugar in the dog is inconstant. In general the concentration tends to become somewhat lowered after anorexia is well established, but extreme grades of hypoglycemia were not encountered (never below 45 mg. per 100 cc.), and severe symptoms of insufficiency and death have been observed repeatedly when the blood sugar level was not significantly lowered. Whether this is peculiar to the dog, and possibly explainable as due to the large amount of accessory medullary tissue found in this species, is a matter of speculation. The tendency to hypoglycemia was more conspicuous in those animals where severe grades of inanition were present. Administration of the cortical hormone alone, without administration of glucose or food to dogs in suprarenal insufficiency has a variable effect on blood sugar concentration. Charts 2 and 3 illustrate these effects in typical experiments. Where the animal does not show marked inanition as Dog 19, a definite rise in blood sugar occurs after a period of hours (fasting), but it does not exceed previous normal levels. Where there is marked inanition (and no doubt very little storage of glycogen) as in Dog F-16, there is no definite effect on the blood sugar. The problem arises as to whether, in the dog, the hypoglycemia observed may not be satisfactorily explained on the basis of the anorexia and inanition alone. We see no substantial basis of support for the theory that the cortical hormone has an important effect in the regulation of the blood sugar level (12), at least in the dog.

Reaction to Insulin.—Marked increase in sensitiveness to insulin is characteristic of dogs without suprarenal glands, although they are maintained in normal condition with cortical hormone. The cortical hormone does not protect the animal against this increased susceptibility, which presumably is due to disturbed function of the medullary tissue. These findings are in agreement with those of Britton and Geiling (7) and of earlier workers mentioned by these authors. Injections of large amounts of the cortical hormone simultaneously do not influence this marked sensitiveness to insulin. (Tables X and XI, and Chart 4 illustrate typical experiments.)

The non-protein nitrogen and other blood constituents can alter rapidly during the terminal stages of insufficiency.

usually the loss of appetite, once established, is complete and permanent.

The characteristic gait of the animal appears only after the earlier symptoms of insufficiency are well established. The first abnormality noted is spasticity of the hind legs which disappears after the animal is released a few minutes outside of the cage. This is followed by a spasticity which persists regardless of exercise. The animal walks haltingly, lurching from side to side, and may finally fall to the floor, unable to rise because of the extreme weakness, particularly in the hind legs.

Vomiting and diarrhea, fluid and sometimes tarry in character, usually appear as later events, often only during the last hours of life. These symptoms in our experience are less constant, for the animal may die before either marked vomiting or diarrhea appears. It is evident that such gastrointestinal reactions may produce a marked effect on the concentration of the blood constituents. It is possible that they may produce chemical changes which have previously been described as characteristic of suprarenal insufficiency. Such may be the drop which has been described in the chlorides and sugar, the concentration of the blood, and the occurrence of acidosis as reported in various studies (7, 8). The clinical picture and the blood changes are practically identical with those seen in the various forms of "shock," as in intestinal obstruction and in cholera.

The fall in body temperature is a constant phenomenon which occurs at about the same time as the fall in basal metabolism and the rise in blood nitrogen. Early in insufficiency the pulse of trained animals, at rest for respiration experiments, is usually elevated. There is usually a terminal drop in pulse rate. Significant changes in systolic blood pressure are a late phenomenon. Irregularities in the pulse rhythm occur in some animals. The mental and nervous symptoms, and hallucinations, which have been described, have not occurred frequently in our animals. A purulent discharge from the eyes is very common in insufficiency, as well as falling out of the hair, increased difficulty in the healing of wounds, and the flaring up of quiescent infections. The inelasticity of the hairy coat is quite striking. In the suprarenalectomized dog during relapse, the skin of the back of the neck remains in an elevated ridge, but slowly relaxing to

EFFECT OF LARGE AMOUNTS OF SUPRARENAL CORTICAL HORMONE ON THE SUPRARENALCTOMIZED DOG IN SUPRARENAL INSUFFICIENCY

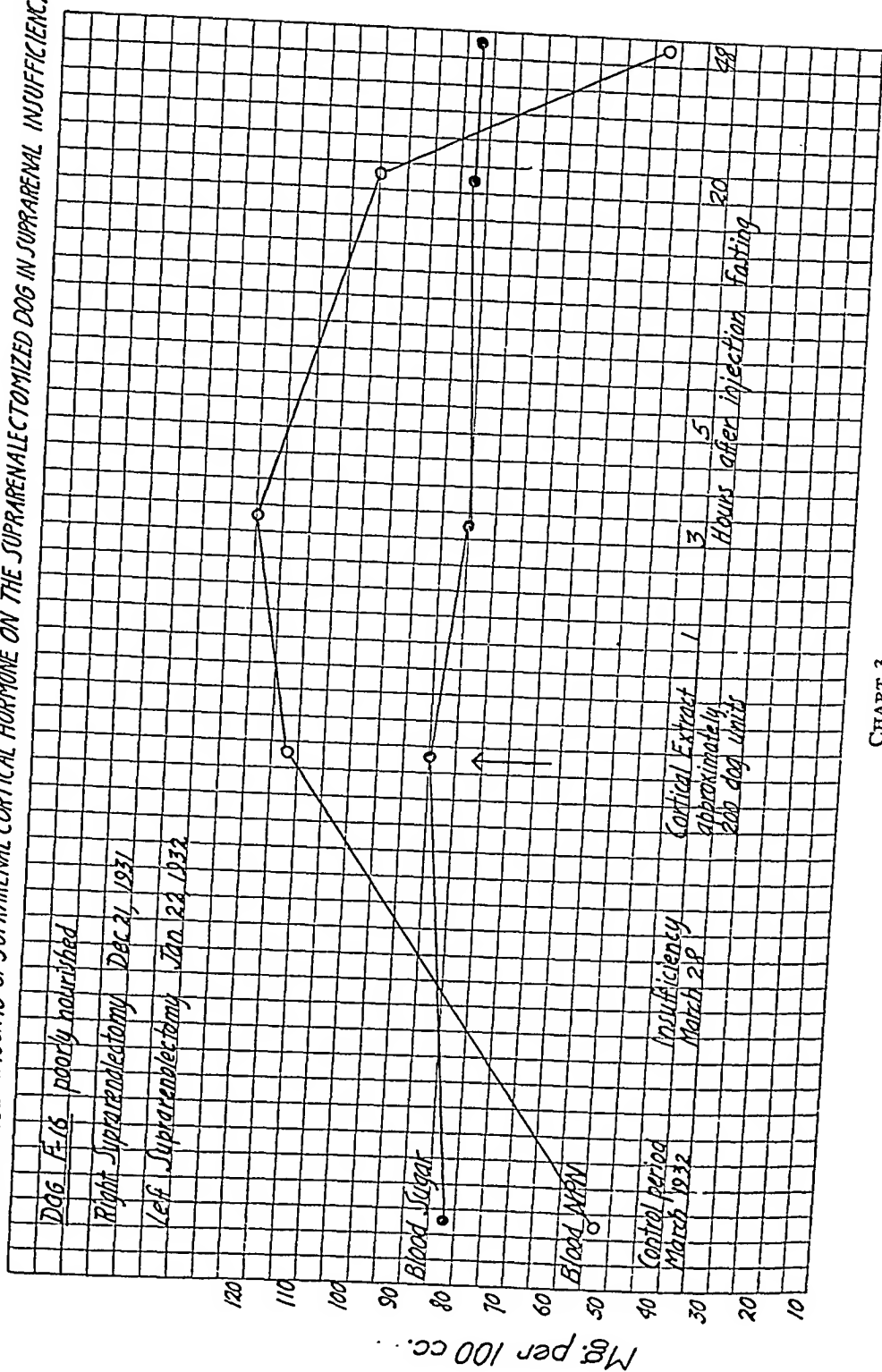


CHART 3

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Mg. per 100 cc.

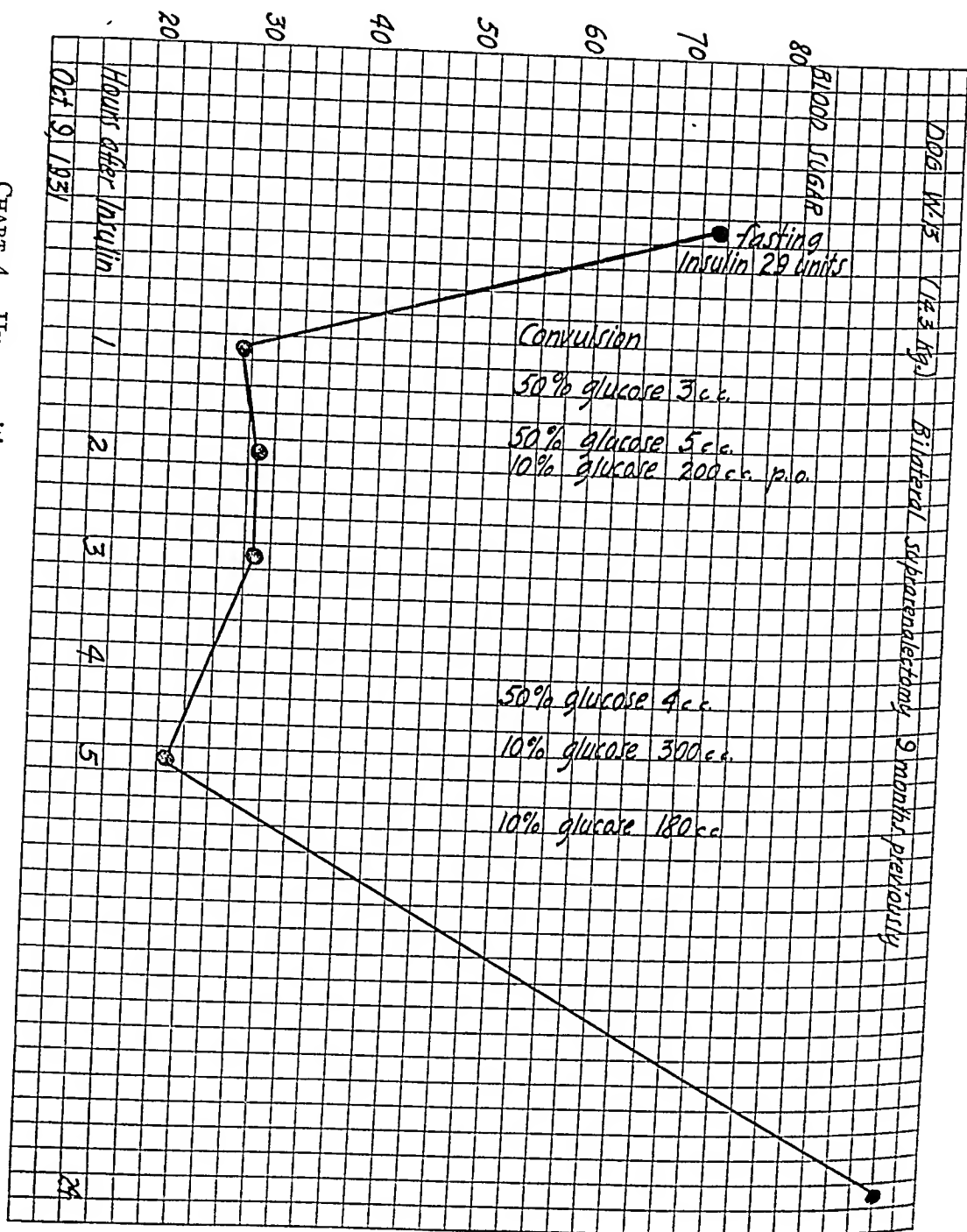


CHART 4. Hypersensitiveness of the suprenalectomized animal to insulin.

not detected until autopsy; distemper, particularly during an epidemic in the late fall of 1931; and the use of insufficient or impotent extract. A number of animals have been killed either to obtain fresh pathological material or to obtain information during the course of certain special tests, such as a study of the toxicity to insulin. The most frequent cause of death has been too great delay in readministration of the extract after withdrawal during studies on the effects of insufficiency. It is very difficult to determine how long a given animal may be permitted to remain without extract. Finally the peculiar chronic inanition described above, following experimentation, has been responsible for a number of our fatalities, and is illustrated in the following typical protocol.

Dog F-31.

	Date	Weight	Non-protein nitrogen
	1932	kg.	mg. per 100 cc.
Right suprarenalectomy	Apr. 11	8.0	30
Left "	" 28	7.9	26
Died	June 15	6.5	175

This animal was used for the study of the hormone-sparing action of oils. On two different occasions coconut oil was given (30 cc. by mouth every day) and the extract allowance reduced to a submaintenance level. In both instances the animal lapsed into insufficiency. During the last period of coconut oil administration the animal lost much weight and failed to recover in spite of large amounts of extract. There was no vomiting or diarrhea. The treatment given was as follows:

Date	Remarks	Weight	Non-protein nitrogen	Extract in dog units (subcutaneous injection)
1932		kg.	mg. per 100 cc.	
June 11	Insufficiency	7.0	88	30
" 12	"	6.7	140	7
" 13	"	6.4	130	600
" 14	"	6.6		200
" 15	"	6.5	175	200
Died 3 hrs. after last injection				

This type of animal, treated with large amounts of extract without improvement, suggests the lack of some factor in addition to the hormone. Autopsy

*Serum Lipids.*²—A considerable number of estimations of serum cholesterol and cholesterol esters, and of serum lipid-soluble phosphorus (lecithin) were made. No definite relation to suprarenal insufficiency or to injections of extract could be established. In many instances the serum lipid-soluble phosphorus concentration tends to be lowered terminally, or in severe insufficiency, while total cholesterol is increased. Sometimes the phosphorus values are very low, but not infrequently normal values are found. We see no correlation between the values for the lipids, which have been studied, and the clinical state of the animals. The blood samples were taken after a fast of 18 hours or more. During insufficiency the fasting period due to the anorexia, may be prolonged.

APPENDIX I

Two monkeys (Bonnet macaque) were suprarenalectomized by Dr. Firor in the fall of 1931 in two stages and injected with cortical extract twice daily thereafter by Dr. L. J. Soffer. Monkey L-19 was suffering from diarrhea prior to the removal of the second suprarenal gland and although 1.1 to 2.6 cc. of extract per kilo was given daily it died on the 11th day. Monkey L-18 was in better condition, the weight being well maintained. It was found dead on the 42nd day after removal of the second suprarenal gland without rise in the blood non-protein nitrogen, and death was attributed to the toxicity of a lot of cortical extract sent us for assay by a pharmaceutical house, which killed two suprarenalectomized dogs during the same period. During the 6 weeks of observation, this animal, Monkey L-18, developed pigmented freckles over the back and chest wall and scrotum, and there may have been slightly increased pigmentation of the lips. Szent-Gyorgi has recently reported that the hexuronic acid compound isolated by him in 1928 from the suprarenal cortex is the hormone effective in preventing the abnormal pigmentation of Addison's disease, although no protocols have as yet been published in support of this announcement. Animals totally deprived of cortical tissue and maintained on the cortical extract only, should be studied for the development of pigmentation. Aside from the observation reported above, which we regard as suggestive only, we have not observed definite evidence of increased pigmentation in any of our animals. Autopsy on both of these monkeys (Dr. Kindell) showed that the suprarenal glands had been completely removed, and no gross accessory suprarenal tissue was detected after careful search.

² These determinations were made with the assistance of Mr. D. J. Verda and Mr. E. A. Pekarek of the Second Year Class. Their aid is hereby acknowledged. Cholesterol and cholesterol esters were determined separately after digitonin precipitation. Lipid-soluble phosphorus was determined after ashing with perhydrol by the Fiske-Subbarow procedure. Alcohol-ether extracts of blood serum were used for these determinations (Bloor).

(d) *Changes in Blood Constituents*

Studies have been made of the behavior of certain blood constituents during suprarenal insufficiency as well as following recovery after the injection of the cortical hormone (Tables VII-IX).

Non-Protein Nitrogen Retention.—Suprarenal insufficiency in the dog is characteristically attended by a rise in the total non-protein nitrogen and the urea nitrogen of the blood. This is usually coincident with, or precedes by a few hours, the onset of anorexia. There is, however, no definite level at which lethal exit occurs, the values found at death in this series ranging from 65 to 240 mg. per 100 cc. The level may depend in part on the physical condition of the animal at the time of withdrawal of the extract, and the speed with which symptoms are induced. The urea nitrogen rises essentially at the same rate as the non-protein nitrogen, so that a very high proportion of the excess of blood non-protein nitrogen is due to increase in the urea. We cannot confirm the existence of a constant or regular increase in the non-urea moiety such as might give clue to the presence of some other nitrogenous body, of possible toxic significance. Only in the terminal stages are appreciable increases in the concentration of creatinine, uric acid, potassium, and inorganic phosphate of the serum to be discovered, and these increases are by no means constant. It seems improbable that this rise in the non-protein nitrogen can be of primary significance in explaining the cause of the relapse. The rise is associated with suppression of urinary nitrogen. In those dogs which void fairly well during the early stages of insufficiency, the rise is not so rapid, but eventually the level may become much higher. The average level of blood non-protein nitrogen at the appearance of the several symptoms noted in a group of dogs is shown (Table VI). Following administration of the cortical extract the drop in non-protein nitrogen usually precedes striking evidences of clinical improvement and may be rapid. It is associated with diuresis and increased excretion of urinary nitrogen (Chart 1). The effect is more prompt and striking in well-nourished animals.

Diminution of Chlorides.—Usually in association with the onset of vomiting and diarrhea one notes a drop, sometimes marked, in the blood chloride concentration. We have not convinced ourselves that this is dissociated from these latter effects or that it is an invariable and characteristic feature of suprarenal insufficiency in the dog. Its occurrence largely depends on the duration of the insufficiency.

Acidosis.—The same conclusion applies to the question of acidosis, which has been reported as occurring during suprarenal insufficiency in cats and in dogs. No changes which can be consistently reproduced have been found in the plasma bicarbonate capacity. The lactic acid of the blood in suprarenalectomized dogs, under properly controlled conditions, postabsorptive, and after prolonged immobilization is constantly very low. The total base of the serum may drop 2 to 4 milimols during suprarenal insufficiency. Acetone bodies are not found in appre-

latter, the deficiency of medullary tissue is also very marked. It is a striking fact that the pathological phenomena singularly unaffected by administration of the cortical hormone in Addison's disease in man (9), namely hypotension, hypoglycemia, and pigmentation, are just those which do not occur as a result of suprarenal deficit in the dog. They are phenomena which a considerable body of experimental evidence associates with disturbance of medullary function and particularly with that of adrenalin. It is a fact that the influence of adrenalin on blood sugar and blood pressure is transient. Recent evidence, however, indicates the probability that not adrenalin but a more powerful related compound is the active pharmacologic agent in the suprarenal gland (10). The abdominal chromaffin body in the dog has been shown to contain as much as 1/12 of the amount of adrenalin present altogether in the suprarenal glands (11).

SUMMARY

1. The methods used in the operation and care of suprarenalectomized dogs are given.
2. The effect of injections of the cortical hormone upon normal and suprarenalectomized dogs is described.
3. Symptoms and chemical studies of suprarenal insufficiency in the dog, following extract withdrawal are described, together with the effects produced by its readministration.
4. The phenomena associated with experimental suprarenal insufficiency are contrasted with those seen in patients with Addison's disease. The identity of the acute symptoms with those of "shock" is pointed out.

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TABLE IX

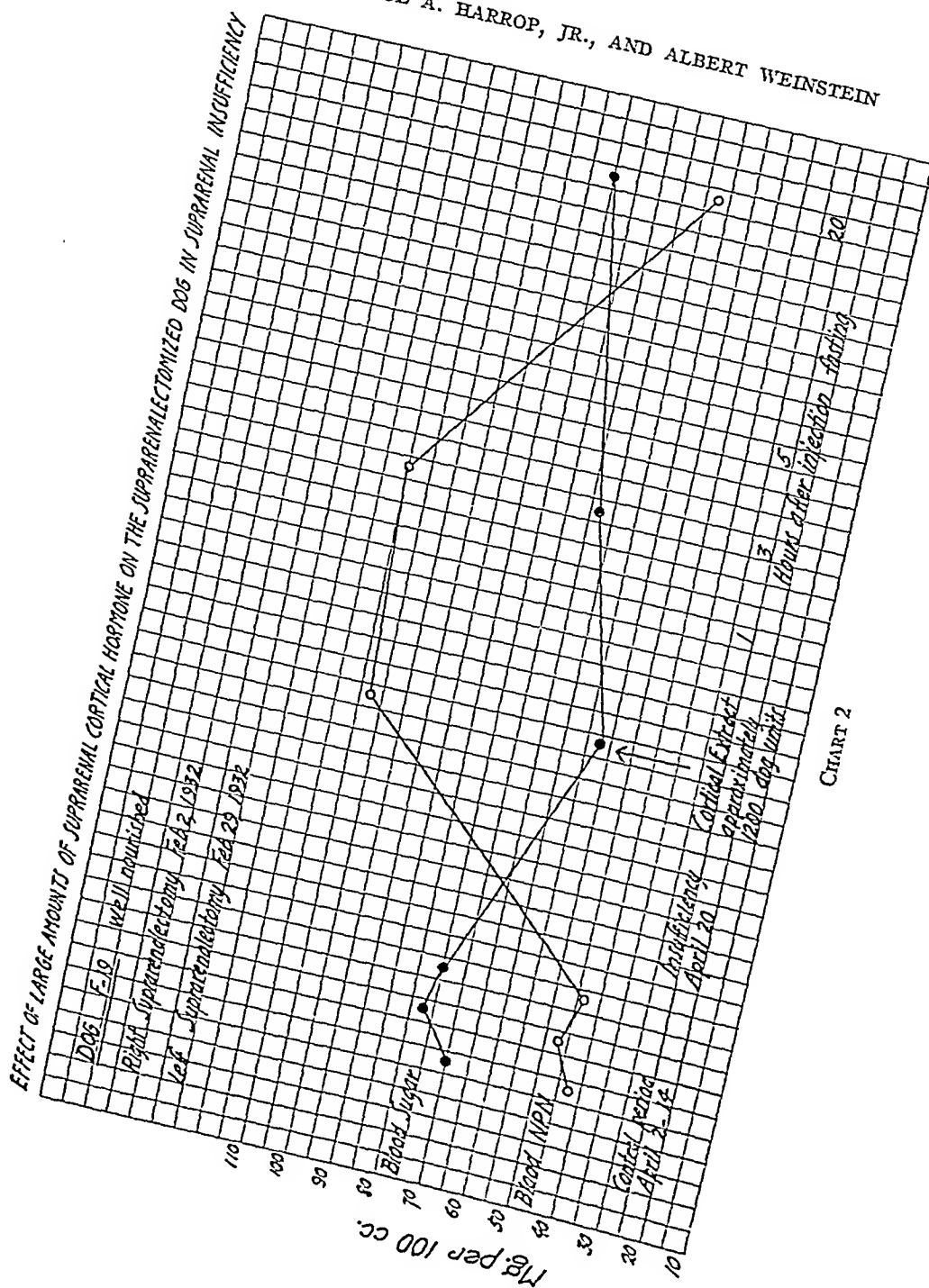
The Changes in Some Chemical Constituents of the Blood (Femoral Artery Blood) Following Extract Withdrawal and during the Progress of the Suprarenal Insufficiency So Produced

Dog W-4, male. Oct. 15, 1930, right suprarenalectomy. Oct. 29, 1930, left suprarenalectomy (Dr. Widenhorn). From Oct. 30, 10 cc. cortical extract daily until Nov. 4, 1930.

Date	Weight kg.	Temperature °F.	Pulse	Remarks	Blood analyses					
					Non-protein nitrogen mg. per 100 cc.	Sugar mg. per 100 cc.	NaCl mg. per 100 cc.	Plasma proteins per cent	Oxygen capacity vol. per cent	Hematocrit per cent
1930										
Nov. 5	10.3	101.2	60	From Nov. 5 to Nov. 15 given 5 cc. cortical extract daily Extract discontinued after Nov. 14	36	72	510	5.4		
" 14	10.4	101.4	80		54	66	530			
" 17 Noon 3 p.m.	10.2	100.6	92		88	60		22.9	46.2	
Nov. 18	10.2	100.0	90	Gait unsteady. Food refused	110	70				
" 19	10.3	99.0	80		148	67	408	23.1	46.5	
" 20	10.0	98.6	40	Electrocardiogram showed idioventricular rhythm. Cannot stand. All food refused. Cortical extract 15 cc.	170	80	402	23.9	52.0	
" 21	97.8	80			190	68		25.0	50.1	
4 p.m.	9 4	97.0	82	Cortical extract 15 cc. at 8 a.m.	250	68	344	6.8	26.0	52.0
				Died just as this blood sam- ple was taken (cardiac puncture)						

In this animal the injections of the hormone were resumed either too late, or in insufficient quantity to prevent a fatal outcome. Autopsy showed complete removal of the suprarenal glands, no accessory tissue, and no evidence of active infection.

The cortical extract used assayed approximately 4 dog units per cc. ciable amounts in the urine. Where acidosis occurs, as has previously been reported, we suggest that it is due to dehydration. The possibility that a retention



K , L , etc., energy levels in the absorbing atom. Other work, notably that of Hanson and Heys (7) on mutations in *Drosophila*, shows a very close relation between the effects of x-radiation and its absorption in the surrounding medium.

The present research was undertaken with the view to determine whether or not there does exist this predicted relation between the bactericidal effect of x-rays, and the absorption of the x-rays in the medium suspending the bacteria; and if the predicted relation does not exist, to determine some of the facts connecting synergistic bactericidal effects, wave lengths of incident x-rays, and their absorptions in the sensitizing media.

Theoretical

In order to reduce results to a strictly quantitative basis, essentially the same method of calculating the number of x-ray quantum hits per bacterium per minute is used as was suggested by Condon and Terrill (8). Suppose in a bacterial suspension, the absorption of *one* quantum by a bacterium is sufficient to kill it. Let A_0 be the original concentration of bacteria. The change with time in the number killed, n , in a layer dx thick at a depth, x , will be proportional to the concentration of living bacteria at time, t , and to the intensity, I , of the x-rays at that depth, so that

$$dn/dt \, dx = \alpha(A_0 - n) I \, dx \quad (1)$$

α is the proportionality constant. Now by the usual absorption law

$$I = I_0 e^{-\mu x}$$

where I_0 is the incident intensity of x-rays, and μ is the absorption coefficient. Hence in a layer D cm. thick

$$dn/dt \int_0^D dx = \alpha(A_0 - n) I_0 \int_0^D e^{-\mu x} dx \quad (2)$$

$$dn/dt = \alpha(A_0 - n) I_0(1 - e^{-\mu D})/\mu D \quad (3)$$

Let

$$\alpha' = I_0 \alpha(1 - e^{-\mu D})/\mu D \quad (4)$$

Then

$$dn/dt = \alpha'(A_0 - n) \quad (5)$$

TABLE X
Hypersensitiveness of the Suprarenalectomized Animal to Insulin
Dog F-7, male, weight 10.0 kilos.

Insulin intravenously per kg.....	Right suprarenal removed—Oct. 14, 1931.		Left suprarenal removed—Nov. 11, 1931. (Dr. Firor)	
	Oct. 9, 1931, intact animal	Nov. 3, 1931, one suprarenal removed	Nov. 23, 1931, both suprarenals removed	
	2 units	2 units	1 unit	
Fasting Hrs. after insulin	Blood sugar	Blood sugar	Blood sugar	
	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	
1	87	86	69	
2	32	32	25	
3	35	32	29	Coma, convulsions
5	37	43	47	Coma, 30 gm. carbohydrate by mouth
7	73	78	95	5 gm. subcutaneously
12		93	95	Coma
Reactions	Decreased activity	Decreased activity	63	Coma, 30 gm. by mouth
				Convulsions—coma—recovery

TABLE XI
Hypersensitiveness of the Suprarenalectomized Animal to Insulin
Dog F-15, male, weight 8.0 kilos.

Insulin intravenously per kg.....	Right suprarenal removed—Dec. 21, 1931.		Left suprarenal removed—Jan. 22, 1932. (Dr. Firor)	
	Jan. 5, 1932, one supra- renal removed	Feb. 2, 1932, both suprarenals removed		
	1 unit	0.5 unit		
Fasting Hrs. after insulin	Blood sugar	Blood sugar		
	mg. per 100 cc.	mg. per 100 cc.		
1	73	63		
2	37	28		Convulsions, 10 gm. glucose intravenously
3	34	245		Coma, convulsions
	32	140		Coma, 25 gm. glucose intra- peritoneally with 0.5 cc.
5	53	408		1/100 adrenalin
7	85	540		Coma, 20 gm. glucose by mouth and 0.5 cc. adrenalin subcutaneously
Reactions	Decreased activity	Died		
				Convulsions—coma—death

In this animal coma persisted in spite of the marked hyperglycemia produced by glucose injections.

Apparatus and Method

A tungsten target x-ray tube was used, permanently connected to a mercury vapor high vacuum system. The plane of the target was set symmetrically with respect to the horizontal and vertical directions, so that ionization measurements might be made on the horizontal portion of the x-ray beam, while bacteria were being irradiated with the vertical portion, of quality similar to that of the horizontal. Ionization measurements were made by means of an open ionization chamber through which passed a narrow pencil of x-rays direct from the target. A short lead connected the electrode inside the chamber to a gold leaf electroscope directly above the chamber. Measurement of absorption coefficients was made by interposing in the beam a filter having parallel sides composed of thin cover-slips 0.63 cm. apart. Intensity of the beam passing through this empty filter was measured by observing the time of fall of the gold leaf across the scale in the eyepiece of the observing telescope. The filter was then filled with the solution under investigation, and the intensity of the beam passing through this thickness of liquid was observed. The ratio of these intensities gave I/I_0 in the absorption equation

$$I/I_0 = e^{-0.63\mu} \quad (13)$$

from which the absorption coefficient μ could be calculated for use in equation (12).

24 hour cultures of *B. coli* were washed from their agar slants with distilled water, filtered, and the suspension diluted to give approximately 2000 bacteria per cc. surviving after 30 minutes, the final dilution being in salt solution of the desired concentration. A series of five Syracuse watch-glasses, 2 cm. diameter, were filled with this suspension to a depth of 0.6 cm. (D of equation (12)), and covered with cover-slips 0.2 mm. in thickness. One dish was retained as a control, while the others were placed 14 inches beneath the target of the x-ray tube. The dishes were rotated so that all would be equally irradiated, and one was removed from the group at the end of each 5 or 10 minute interval. When all had been irradiated, 1 cc. of suspension was drawn from each dish and placed in test-tubes containing 5 cc. of distilled water, giving suspensions of approximately 350 bacteria per cc. These suspensions were plated in quadruplicate, being plated in order, then reverse order, and repeat. This method negated the lethal effects of strong salt solutions with passage of time during the plating process. The plates were incubated 24 to 48 hours, counted, and the percentages of surviving bacteria determined. Three such sets were generally made for each solution, the surviving percentages averaged, and plotted against time of irradiation.

Peculiarly enough, the preponderance of data was definitely in favor of curves of the two-hit type, having the equation

$$A = A_0 e^{-\alpha' t} (1 + \alpha' t)$$

No. P-1 was a male dog with infections at the sites of both operative incisions for removal of the suprarenals, which, however, seemed to heal well. This animal was used for assay of the cortical extract. Feb. 6, 1931, on adequate doses it was doing well and was maintaining weight. 18 days later, after the extract was reduced to 0.1 cc. per kilo per day for 3 days, the dog went into insufficiency from which it recovered with the use of large amounts of extract (2 cc. per kilo for 3 days and 1 cc. per kilo for the next 2 days). The dog then seemed in good condition and extract was omitted for only 24 hours. In the morning of the following day (Mar. 12) it seemed well, although it had not eaten. The temperature was 101.2°. The dog was permitted to exercise in the yard during the morning, but at 2.30 p.m. vomited, and at 4 p.m. when removed from the cage had watery diarrhea and could not stand. Temperature 96°, pulse 110. Glucose and saline injections were given intraperitoneally, as well as 2 cc. of extract per kilo, but the dog died during the night. The abrupt changes in the (arterial) blood findings follow.

	Blood constituents						
	Non-protein nitrogen	Urea nitrogen	Sugar	Creatinine	Inorganic serum phosphorus	Serum potassium	Serum base
1931	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	m.-eq. per liter
Mar. 11, 9 a.m. Condition seemed good but not eating. No extract given	60	35	60	1.5	7.5	25	152.6
Mar. 12, 4 p.m. Collapse followed exercise. Blood sample taken before administration of glucose, fluids, or extract	104	82	65	2.3	13.2	42	147.6

This animal illustrates the abrupt collapse which may follow exercise when the dog is in early insufficiency, the rapid change which may take place in various blood constituents, *without* change in the sugar concentration, and the usual terminal rise in serum potassium and inorganic phosphates. When the animal has been deprived of extract in sufficient dosage over a considerable period, insufficiency is very rapidly induced, and if it is allowed to advance too far, as in this case, a fatal result occurs in spite of energetic measures. Extract from dissected cortex was used throughout this experiment and the assay value was 6 units per cc.

Apparatus and Method

A tungsten target x-ray tube was used, permanently connected to a mercury vapor high vacuum system. The plane of the target was set symmetrically with respect to the horizontal and vertical directions, so that ionization measurements might be made on the horizontal portion of the x-ray beam, while bacteria were being irradiated with the vertical portion, of quality similar to that of the horizontal. Ionization measurements were made by means of an open ionization chamber through which passed a narrow pencil of x-rays direct from the target. A short lead connected the electrode inside the chamber to a gold leaf electroscope directly above the chamber. Measurement of absorption coefficients was made by interposing in the beam a filter having parallel sides composed of thin cover-slips 0.63 cm. apart. Intensity of the beam passing through this empty filter was measured by observing the time of fall of the gold leaf across the scale in the eyepiece of the observing telescope. The filter was then filled with the solution under investigation, and the intensity of the beam passing through this thickness of liquid was observed. The ratio of these intensities gave I/I_0 in the absorption equation

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Peculiarly enough, the preponderance of data was definitely in favor of curves of the two-hit type, having the equation

$$A = A_0 e^{-\alpha' t} (1 + \alpha' t)$$

DISCUSSION

A comparison of the phenomena observed clinically during the crisis of Addison's disease with those of suprarenal insufficiency in dogs after withdrawal of extract, reveals striking similarities, as well as differences which must be equally significant. The points of resemblance are the extreme muscular weakness, the anorexia, vomiting and diarrhea, the lowered general bodily activity associated with lowered body temperature, the lowered general metabolism, the increased blood concentration, and the characteristic increase in blood non-protein and urea nitrogen. The serum potassium and inorganic phosphate characteristically increase and the total serum base falls. It seems clear that all of these phenomena are dependent directly or indirectly on lack of the cortical hormone. With the possible exception of the extreme muscular asthenia, they are also characteristic of "shock" as produced by a variety of causes both in man and in the experimental animal.

On the other hand, proper dosage of cortical extract in the dog appears to maintain normal nutrition and weight and to preserve life indefinitely. The blood pressure is normal, there is no lowering of the blood sugar, no increased pigmentation, and no change in the basal respiratory metabolism. There is an increased sensitivity to insulin and to thyroid extract.

In contrast, the patient with advanced Addison's disease, even when given large amounts of a potent preparation of cortical extract continues to have hypotension, hypoglycemia, and no striking amelioration in the pigmentation which is characteristic of the disease and almost always present. He is in general very sensitive to insulin and to the administration of thyroid extract. The effect on weight and nutrition is variable and often very slight. The basal metabolic rate is lowered in most patients.

That the differences between the human disease and the effects produced by the experimental deficit can be ascribed to tuberculous infection, present elsewhere in the body in Addison's disease, is at once disproved by the fact that the salient clinical features of the disease are just as characteristic of the cases due to atrophy of the suprarenal cortex where no tuberculosis whatever is present. In the

EXPERIMENTAL RESULTS

Experimental data and calculated results are shown in Tables I to IV.

TABLE I

Samples of Original Data Illustrating the Determination of the Number of Effective Hits per Bacterium per Minute

<i>B. coli</i> suspended in	Time of irradiation	Survival (A/A_0) average for three determinations	α' calculated from one-hit equation $A/A_0 = e^{-\alpha' t}$	α' calculated from two-hit equation $A/A_0 = e^{-\alpha' t}$ ($1 + \alpha' t$)
	<i>min.</i>	<i>per cent</i>		
Distilled water	5	94.8	0.011	0.071
	10	82.6	0.019	0.075
	15	70.2	0.024	0.073
	20	59.3	0.026	0.070
Mean.....				0.072 \pm 0.001
$10^{-1.04}$ M $\text{Ba}(\text{NO}_3)_2$	5	95.0	0.010	0.068
	10	84.3	0.017	0.070
	15	74.2	0.020	0.065
	20	63.0	0.023	0.064
Mean.....				0.067 \pm 0.001
$10^{-1.04}$ M $\text{Pb}(\text{NO}_3)_2$	5	86.2	0.030	0.129
	10	63.2	0.046	0.129
	15	39.5	0.062	0.136
	20	31.9	0.057	0.118
Mean.....				0.129 \pm 0.002

TABLE II

Effects of X-Rays on B. coli in Various Solutions

Tube operated at medium voltage. 10 ma., filter: 0.033 cm. Al.

Solution	Initial toxicity	μ	K (equation (12))	α'	$\alpha = \alpha'/K$
	<i>per cent</i>				
H_2O	0.0	0.80	0.80	0.072	0.090
$10^{-1.04}$ M KBr	0.0	0.99	0.75	0.067	0.090
$10^{-1.04}$ M $\text{Sr}(\text{NO}_3)_2$	10	1.07	0.74	0.067	0.091
$10^{-1.04}$ M $\text{Ba}(\text{NO}_3)_2$	10	1.15	0.72	0.067	0.093
$10^{-1.04}$ M $\text{Pb}(\text{NO}_3)_2$	90	1.65	0.63	0.129	0.205

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Fig. 1 shows the data of Table II in graphical form, with α plotted against μ . A very similar curve is obtained if α be plotted against the molecular weight of the various salts, or (in the case of the nitrates) against the atomic number of the positive ion. Fig. 2 shows the data of Table III plotted as α against log molarity, while Fig. 3 shows α plotted against log μ , for both $\text{Pb}(\text{NO}_3)_2$ and KBr .

DISCUSSION AND CONCLUSIONS

Figs. 1, 2, and 3 do not lend themselves readily to the formation of any quantitative law, except at low concentrations, where there seems

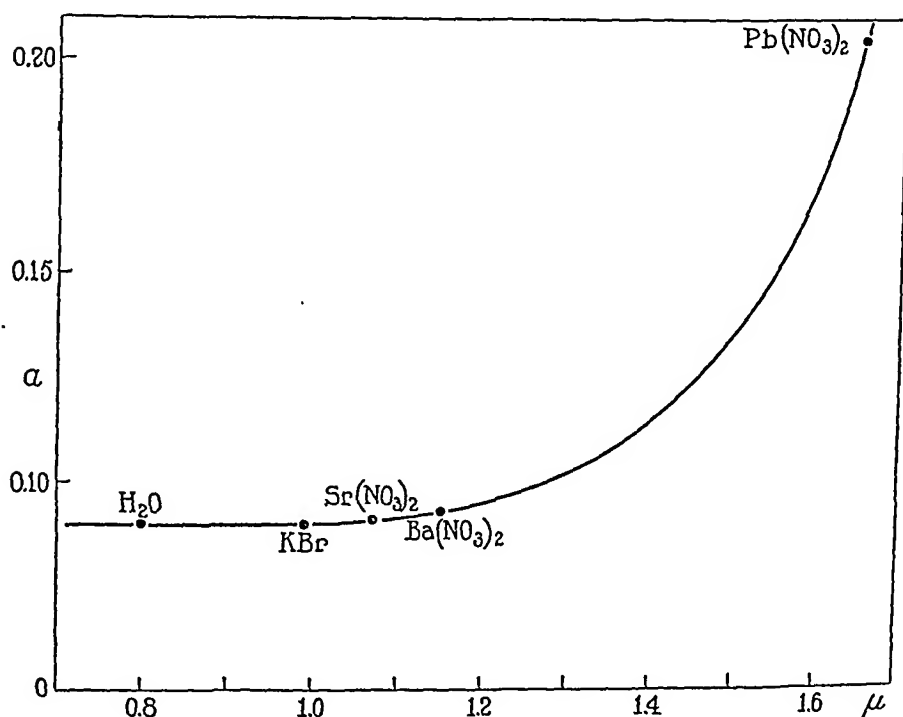


FIG. 1. Number of effective hits per minute per bacterium as a function of the absorption coefficients of $10^{-1.04}$ molar concentrations of various salts.

to be a linear relation between α and μ . They indicate very definitely, however, that some fairly high value of the absorption coefficient must be reached before there is any great increase in the effect of x-rays when used in conjunction with x-ray fluorescent salts. In fact, as an inspection of the values of α' in Table II will show, the actual number of hits per bacterium per minute in some salt solutions is less than in water, due to the shielding effect of the salt. Only in the case of

ENHANCED LETHAL EFFECTS OF X-RAYS ON BACILLUS COLI IN THE PRESENCE OF INORGANIC SALTS

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INTRODUCTION

It is well known that living cells which are subjected to the effects of x-rays will have those effects increased if irradiated in the presence of certain compounds, as salts, dyes, and stains. Ellinger and Gans (1) found the effects of x-rays to be greatly increased by the presence of thorium salts. Ghilarducci and Milani (2) and Baldwin (3) have reported that the presence of fluorescent dyes enhances the effect of x-rays on a variety of living cells. Anderson (4) has reported that putting afflicted animals on a high salt diet increases the susceptibility of their tumors to x-rays, while Sperti and Norris (5) claim a similar effect on transplantable tumors in the presence of caesium iodide and x-rays.

Norris (6) has shown the effect to be truly synergistic, in that when bacteria are irradiated by x-rays in the presence of salt solutions, the effects are greater than when the bacteria are subjected to x-rays and salt solution successively. Norris also suggests the possibility of obtaining still greater lethal effects by using a wave length of x-rays corresponding to one of the deeper energy levels in the molecule of salt used, such as the K level of the metallic ion. If this be possible, the synergistic effects can be related directly to the absorption of x-rays by the salt dissolved in the water suspension of bacteria, for sharp breaks appear in the curves for x-ray absorption in various substances, these breaks occurring when the wave length of the x-rays absorbed is such as to have a quantum energy exactly equal to the

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sensitizers is to increase the amount of energy absorbed within the cells, or some special part of the cells, and the effectiveness of the sensitizer will vary with this factor. For this reason it may be of considerable importance whether or not the sensitizer penetrates into the cell, or possibly becomes located in some particular part of the cell in high concentration. It may be that the initial toxicity of the salt gives some measure of this penetration, even for the cells which survive.

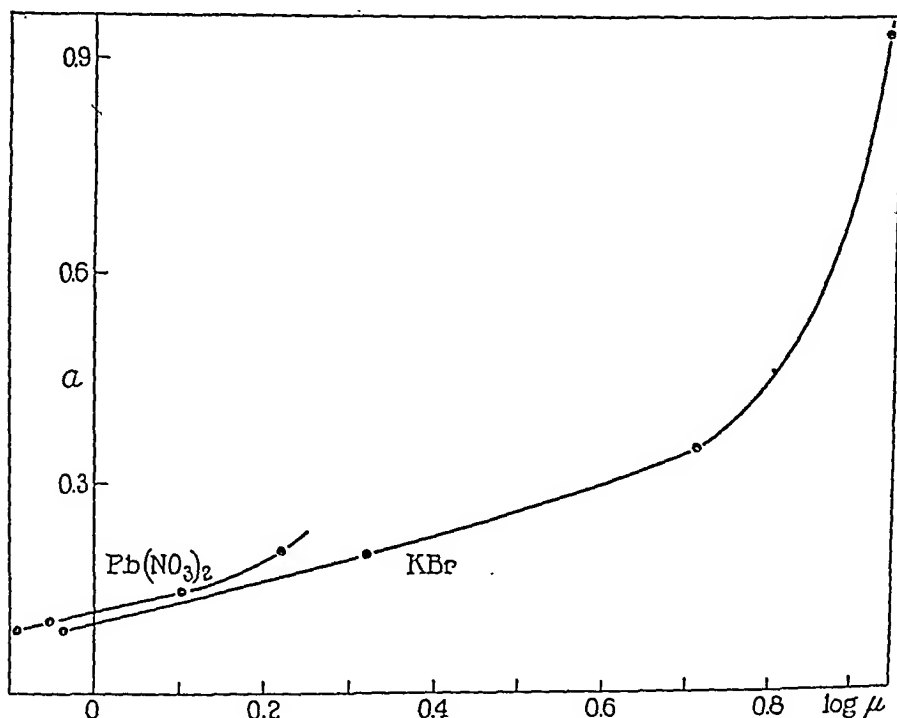


FIG. 3. Number of effective hits per minute per bacterium, α , plotted as a function of the log of the absorption coefficient, μ , for increasing concentrations of $\text{Pb}(\text{NO}_3)_2$ and KBr .

Then, there is to be considered the possible secondary chemical effects of the sensitizing salts. The same primary injury produced by the absorption of x-ray quanta or photoelectrons might lead to different results in the presence, for example, of potassium, calcium, and lead, due to their chemical effects on protoplasm.

Results of the experiment with various qualities of radiation (Table IV) present what at first seems to be a surprising situation in that, for

and upon integrating, we obtain

$$(A_0 - n)/A_0 = e^{-\alpha' t} \quad (6)$$

where $(A_0 - n)/A_0$ is the fraction surviving after irradiation for a time t . Suppose, however, that *two* hits are required to kill, and let N be the number killed (or hit twice). Then

$$dN/dt \, dz = \alpha(n - N) \, I \, dz \quad (7)$$

From (6)

$$n = A_0 (1 - e^{-\alpha' t})$$

Hence

$$\begin{aligned} dN/dt &= \alpha'(n - N) \\ &= \alpha' A_0 (1 - e^{-\alpha' t}) - \alpha' N \end{aligned}$$

or

$$dN/dt + \alpha' N = \alpha' A_0 (1 - e^{-\alpha' t}) \quad (8)$$

which is an ordinary linear differential equation of the form

$$dy/dt + Py = Q$$

having the solution

$$y = e^{-\int P \, dt} \left(\int Q e^{\int P \, dt} + C \right) \quad (9)$$

Solving (8) thus, we obtain the surviving fraction as

$$(A_0 - N)/A_0 = e^{-\alpha' t} (1 + \alpha' t) \quad (10)$$

Similarly, it may be shown that if r hits are required to kill, the surviving fraction will be given by

$$(A_0 - R)/A_0 = e^{-\alpha' t} [1 + \alpha' t + (\alpha' t)^2/2! + (\alpha' t)^3/3! + \dots + (\alpha' t)^{r-1}/(r-1)!] \quad (11)$$

This expression is the same as that developed by Condon and Terrill (8) and Wyckoff and Rivers (9) on the basis of probability theory. It is to be remembered, however, that the α' determined from the experimental curves is proportional to both I_0 and $(1 - e^{-\mu D})/\mu D$, so that if I_0 remains constant, observed values must be divided by the "shielding constant," K , where

$$K = (1 - e^{-\mu D})/\mu D \quad (12)$$

in order to obtain a comparable number of effective hits per bacterium per minute, α .

tested by the use of monochromatic x-rays, a test not so easy to make because of the difficulty of obtaining monochromatic rays in sufficient intensity.

The limited data of Table IV (last column) show that while α is the same for water for all wave lengths, it is considerably greater when the sensitizers are used, and especially greater for the shorter wave lengths. Thus, when used in conjunction with sensitizers, the shorter and more practical rays are also the more efficacious, despite the shielding effects of the sensitizers and the smaller μ of the solutions for the shorter wave lengths. From a practical point of view, this phase of the problem might profitably bear a more extended investigation.

In conclusion, the author wishes to express his thanks and appreciation to Prof. S. C. Brooks for suggesting the problem, and for his continued interest and help during the progress of the work; to Dr. M. M. Brooks for her interest and many suggestions; and to Mrs. W. D. Claus for her faithful assistance in the laborious task of plating and counting bacteria.

SUMMARY

1. When *B. coli* are irradiated by x-rays in a series of salt solutions of tenth molar concentration, the synergistic effect does not become appreciable until heavy salts are used.
2. When $\text{Pb}(\text{NO}_3)_2$ or KBr solutions are used in increasing concentrations, the synergistic effect is not appreciable until large concentrations or absorption coefficients are reached, whereupon the effect increases sharply. Thus the number of effective hits per bacterium per minute, α , is a function of the absorption coefficient, μ .
3. The sharp increase in α does not occur at the same concentration, or same μ , for $\text{Pb}(\text{NO}_3)_2$ and for KBr. Thus α is a function of the nature of the salt, or possibly of the penetration of the salt into the cell, some measure of which may be obtained from the initial toxicity of the solution.
4. For a given solution, α increases as the wave length λ of the x-rays decreases, although μ decreases by the same process as the cube of λ . Thus α is a function of λ to some power greater than the cube.
5. A possible mechanism whereby the synergistic effect takes place

whereas Wyckoff and Rivers (9) obtained curves of the one-hit type.¹ From these curves, α' was calculated for each point, all values averaged, and the average α' divided by the shielding constant of equation (12) to obtain α . Each value of α given in the tables is thus the result of counts on some 15,000 bacteria. The probable error in α' , in general, is about 5 per cent or less. (Illustrative data are given in Table I.)

The number of effective hits per bacterium per minute, α , was determined for *B. coli* in distilled water and in $10^{-1.04}$ molar solutions of KBr, Sr(NO₃)₂, Ba(NO₃)₂, and Pb(NO₃)₂. Nitrates were chosen so that the negative ion would possess an absorption coefficient negligible with respect to that of the positive ion. (The presence here of KBr is incidental to the experiments of Table III.) Results are given in Table II. α was also determined for various concentrations of Pb(NO₃)₂ and KBr. Because of the high toxicity of heavy metal salts, concentrations of Pb(NO₃)₂ greater than tenth molar were not practical, but KBr could be used in much greater concentrations. Results are given in Table III. The approximate initial toxicities of the salt solutions used are also given in these two tables.

In these experiments, the voltage on the x-ray tube was such as to give, with an aluminum filter 0.033 cm. thick in the beam, an "effective wave length" of (atomic absorption coefficient in lead corresponding to) 0.45 \AA . The tube was operated at 10 ma., working distance 14 inches from the target.

In another set of experiments, $10^{-1.04}$ molar Pb(NO₃)₂ and $10^{0.30}$ molar KBr were used with varying hardness of x-rays. In one case, the voltage on the x-ray tube was just sufficient to give a working intensity of radiation at 10 ma., with no filter in the beam. In another case, the voltage was stepped up as high as possible, and a filter of 0.175 cm. of aluminum used in the beam to absorb the softer constituents. Because it was impractical to obtain equal intensities for all qualities of radiation, and in order to make the values comparable, α was determined for *B. coli* in distilled water for each voltage, and the α 's for Pb(NO₃)₂ and KBr brought to a common basis by proportion. The results of these experiments are shown in Table IV. Absorption coefficients were determined for all solutions used, and intensities were checked by ionization measurements from time to time during the experiments to make sure that no variation was taking place.

¹ The only obvious experimental difference is that the bacteria of this experiment were irradiated, then plated, while those of Wyckoff's experiment were plated, then irradiated. This difference in experimental procedure, however, hardly seems to offer an adequate explanation for the difference in type of curve obtained, and suggests that some factor other than radiation may be operative, as for example the distribution of sensitivity in the bacterial population, which varies with the age of the culture. Thus some doubt may be thrown upon the identification of α' with the number of effective hits per bacterium per minute, but because α' is a constant for a given set of conditions of irradiation, and not a function of time (as is survival), it is a convenient quantity to use for the comparison of effects, whatever its precise significance.

TABLE III
Effects of X-Rays on B. coli in Pb(NO₃)₂ and KBr Solutions of Various Concentrations
 Tube operated at medium voltage, 10 ma., filter: 0.033 cm. Al.

Solution	Initial toxicity per cent	μ	K (equation (12))	α'	$\alpha = \alpha'/K$
$10^{-1.04}$ M Pb(NO ₃) ₂	90	1.65	0.63	0.129	0.205
$10^{-1.30}$ M Pb(NO ₃) ₂	80	1.27	0.70	0.102	0.146
$10^{-2.01}$ M Pb(NO ₃) ₂	25	0.89	0.77	0.079	0.103
$10^{-3.04}$ M Pb(NO ₃) ₂	10	0.81	0.79	0.074	0.094
$10^{-\infty}$ M Pb(NO ₃) ₂	0.0	0.80	0.80	0.072	0.090
$10^{0.56}$ M KBr	50	8.70	0.19	0.178	0.937
$10^{0.20}$ M KBr	15	5.10	0.31	0.109	0.352
$10^{0.00}$ M KBr	5	2.90	0.47	0.093	0.200
$10^{-1.04}$ M KBr	0.0	0.99	0.75	0.067	0.090
$10^{-\infty}$ M KBr	0.0	0.80	0.80	0.072	0.090

TABLE IV
Effect of Changing Hardness of X-Rays on B. coli in Salt Solutions

Solution	μ^*	K (equation (12))	α'	$\alpha = \alpha'/K$	α adjusted to basis of 0.070 for water
H ₂ O	1.18	0.72	0.082	0.114	0.090
$10^{-1.3}$ M Pb(NO ₃) ₂	2.00	0.58	0.082	0.141	0.111
$10^{0.3}$ M KBr	8.08	0.20	0.075	0.375	0.296
H ₂ O	μ^\dagger				
$10^{-1.3}$ M Pb(NO ₃) ₂	0.80	0.80	0.072	0.090	0.090
$10^{0.3}$ M KBr	1.27	0.70	0.102	0.146	0.146
	5.10	0.31	0.109	0.352	0.352
H ₂ O	μ^\ddagger				
$10^{-1.3}$ M Pb(NO ₃) ₂	0.48	0.87	0.060	0.069	0.070
$10^{0.3}$ M KBr	0.93	0.77	0.089	0.116	0.151
	3.56	0.41	0.133	0.324	0.423

* Low voltage, 10 ma., no filter in beam.

† Medium voltage, 10 ma., filter: 0.033 cm. Al.

‡ High voltage, 10 ma., filter: 0.175 cm. Al.

and that in the absence of other factors, or more prolonged stimulation, the stored protein was catabolized.

The effect of brief treatments with extracts of beef anterior pituitary on the nitrogen balance and urine nitrogen partition of dogs was therefore studied. Observations were also made on the effect of such extracts on water balance, body weight, and lactation. On one of the animals, respiration experiments showing the effects of the extracts on heat production and on the metabolism of carbohydrate, fat, and protein were carried out. The extracts were similar to those of Teel (6), and consisted of the globulin fraction, salted out by means of sodium sulfate, from partially neutralized alkaline extracts of beef anterior pituitary. Preparations from the pituitary of sheep have been used by Corner (7) and by Nelson and Pfiffner (8) in studies of lactation in rabbits and guinea pigs. Details concerning the extracts are included in the following section.

EXPERIMENTAL

Animals.—Three adult bitches were used as experimental animals. One of these, Dog 2, was a young virgin weighing 15 kilos. The other two, Dogs 1 and 4, were older animals which weighed 21 and 22 kilos respectively, and had apparently borne litters. All of the animals were kept in the metabolism cages and on the desired diet for several weeks before experiments were begun. With about 15 minutes of exercise daily, after catheterization, they have kept in excellent condition on the experimental regime during more than a year.

Diets.—The diets consisted of beef heart, soda crackers, and bone meal. Grinding the three constituents together in a mechanically driven meat grinder greatly simplified analysis and weighing. 4 pounds of trimmed, ground beef heart, mixed with an equal weight of ground crackers and 200 gm. of bone meal, and again ground four times, yield a mixture which has very desirable properties. The cracker meal absorbs the excess of water in the beef heart, hence the mixture becomes more suitable for analysis. 4 gm. samples yield good duplicate values for nitrogen. The mixture keeps for 2 to 3 weeks, and remains unchanged in nitrogen content if it is placed in air-tight tin cans and kept in the refrigerator. In experiments in which a higher carbohydrate intake was desired, one part of trimmed beef heart and two parts of cracker meal were used in preparing the mixture. If necessary, the amount of bone meal can be doubled. When beef heart alone was used, a number of carefully trimmed hearts were ground and well mixed. Approximately 7 gm. samples were then analyzed for nitrogen. The remainder was put up in 200 gm. lots, which were wrapped in wax paper and frozen until required for feeding.

Methods.—Daily urine periods were terminated by catheterizing and washing

$10^{-1.04}$ molar $\text{Pb}(\text{NO}_3)_2$ with a relatively high value of μ does the synergistic effect become sufficiently great to overcome the shielding effect. (It would be very desirable to have another point on the curve between $\text{Ba}(\text{NO}_3)_2$ and $\text{Pb}(\text{NO}_3)_2$, but no salt could be found in this region which was not entirely lethal at a concentration of $10^{-1.04}$ molar.) This sharp upward trend in α as μ increases is again in evidence with increasing concentrations of $\text{Pb}(\text{NO}_3)_2$ and KBr , but occurs

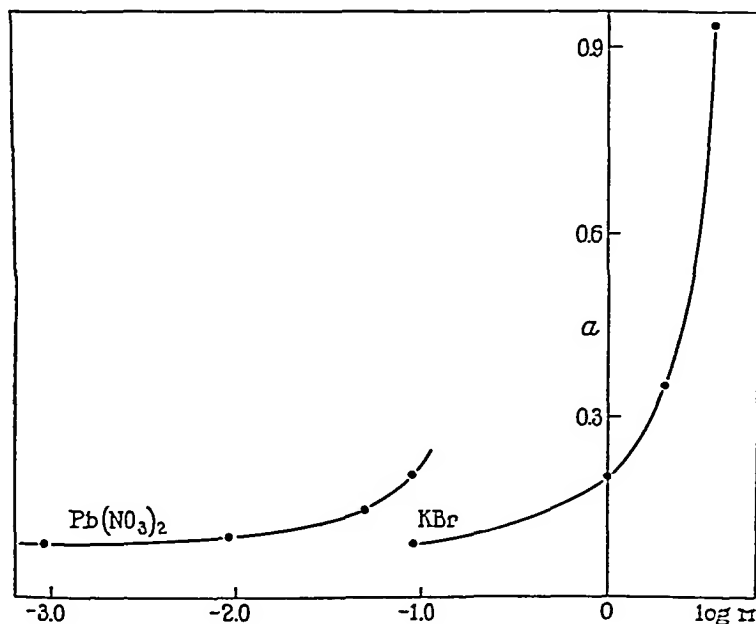


FIG. 2. Change in the number of effective hits per minute per bacterium with increasing concentrations of $\text{Pb}(\text{NO}_3)_2$ and KBr ; α plotted as a function of log molarity.

at widely separated values of μ for the two salts. Let us call the difference between α for salt solutions and α for water, $\Delta\alpha$. Then $\Delta\alpha$ must be not only a function of μ , but also of the nature of the sensitizer, with lead and presumably other heavy salts being relatively much more efficient. It is entirely conceivable, however, that elements might exist with low absorption and high synergistic effect, and *vice versa*, and data from the restricted group here studied are not sufficient to prove the contrary. The principal object to be attained by the use of

6 portions of 10 cc. or less, the injections being made into the loose subcutaneous tissue of the abdomen and flank. They were well tolerated. After intraperitoneal injections of 50 cc., the animals commonly delay taking their food for several hours, but consume it as usual in the course of the day.

RESULTS

Nitrogen Balance.—Preliminary experiments showed that intraperitoneal or subcutaneous injections of the extract produced a fall in urine nitrogen, while control injections of physiological salt solution, of boiled extract, or of highly diluted blood serum containing an amount of protein similar to that in the extracts, had negligible effects. The experiment recorded in Table I was therefore carried out to determine whether the fall in urine nitrogen was due to failure of absorption, to an effect of the extracts on the kidneys, or to storage of nitrogen. The diet consisted of 400 gm. of beef heart-cracker meal mixture, containing 10.11 gm. of nitrogen. During the control period from November 5 to 11, two 50 cc. injections of boiled extract produced no marked change in urine nitrogen. Similar treatment with unboiled extract on November 12 and 13 resulted in a marked drop in urine nitrogen to 4.73 gm. per day, from an original average level of 9.1 gm. per day. In 6 days the original level was reached again and passed, but a second similar treatment lowered the urine nitrogen even more markedly to 3.97 gm. per day. Feces analyses were carried out daily, since the animal had one large movement per day. The results indicate that absorption was not interfered with, and the fall in non-protein nitrogen of the blood excludes the possibility that the extracts damaged the kidneys. Hence the decrease of urine nitrogen must be due to storage of nitrogen in the tissues. The extent of this storage is quite considerable, since the average daily excretion is 1 to 1.5 gm. below the control level for 10 to 11 day periods following injections. The high control level for non-protein nitrogen of the blood was due to the high protein diet, not to renal insufficiency. During the final control period cod liver oil and tomato juice were added to the diet, but no sudden changes of nitrogen excretion occurred. These changes are at times observed if a missing food factor is given to an animal that is on a deficient diet.

The first part of Table II is the record of an experiment in which the fall in nitrogen excretion was not observed.

both $\text{Pb}(\text{NO}_3)_2$ and KBr , the number of effective hits, α , decreases as the absorption coefficient μ increases, in a manner contrary to expectations based on the two previous experiments. Obviously, however, a change in absorption due to a change in wave length would involve a wholly different set of subatomic phenomena than a change in absorption due to a change in concentration of sensitizer. The experimental result is, moreover, qualitatively in accord with the findings of Wyckoff (10), who obtained larger "sensitive volumes" (or greater ratios of effective hits to total absorptions) for shorter wave lengths. That is, the number of effective hits as determined from survival curves is less than the number of quantum absorptions as calculated from the number of incident quanta, the size of the organisms, and the absorption coefficient of protoplasm. For *B. coli*, Wyckoff found the lethal efficiency of x-ray absorptions to be of the order of 20 per cent, and that the efficiency increases with decreasing wave length. A possible explanation of this phenomenon is that when the cells occupy a relatively small fraction of the total volume irradiated, the longer ionization tracks produced by the photoelectrons of larger quanta have a better chance of being included in the sensitive volumes of the cells, and thus lead to a greater efficiency of the radiation. In fact, one would expect on this basis, that the efficiency would be proportional to the square of the energy of the ejected photoelectrons, but unfortunately this possibility cannot be checked numerically from the data of this paper, because general rather than monochromatic radiation has been used. Thus, α becomes a function also of wave length, λ , in such a way that the decrease of $\Delta\alpha$ with increasing λ more than outweighs the effect of an increasing μ . Data from physical tables show that for a given substance, μ increases as the cube of λ (within limits of λ); hence $\Delta\alpha$ must be a function of λ to some power greater than the cube, as well as being a function of the nature of the salt.

The above discussion may furnish another clew to the explanation of the observed increase of α as heavier salts are used. With a heterogeneous beam such as was used in this experiment, the softer rays are absorbed before the harder ones, but as μ increases, as for $\text{Pb}(\text{NO}_3)_2$, a larger proportion of hard (and hence more efficient) rays are absorbed, giving the effect of efficiency increasing more rapidly than the absorption coefficient. Here again, the hypothesis could only be

Urine collections and analyses were made daily, but due to the length of the experiment the daily averages for the week are recorded. The animal, which weighed three-fourths as much as the preceding one, was receiving exactly one-half as much food,—200 gm. of beef heart-cracker meal mixture, containing 5.02 gm. of nitrogen. The injections were given on the same dates as in the preceding experiment, and the same lot of extract was used. The results were negative until 200 gm. of beef heart was added to the diet, increasing the nitrogen intake to 10.35 gm. per day. Injection of extract on Jan. 1 and 2 then produced a defi-

TABLE II

Showing That the Effect of Anterior Pituitary Extract on the Nitrogen Balance Varies with the Diet

Dog 2.

Period	Daily average				Extract given subcutaneously
	Food N	Urine N	Feces N	Urine volume	
1931-32	gm.	gm.	gm.	cc.	
Nov. 5-11	5.02	3.79		329	None
12-19	5.02	3.73		348	50 cc. No. 094891A, daily Nov. 12 and 13
20-26	5.02	3.98		261	None
27-Dec. 6	5.02	3.83	0.71	471	50 cc. No. 094891A, daily Nov. 27 and 28
Dec. 7-13	5.02	3.98	0.68	319	None
28-31	10.35	8.37		298	None
Jan. 1- 7	10.35	7.56		492	50 cc. No. 094900A, daily Jan. 1 and 2
8-14	10.35	9.45		293	None
15-21	10.35	9.74		297	None
22-27	10.35	8.96		290	None
28-Feb. 2	10.35	8.54		248	None

nite fall in nitrogen excretion. During the week following injection of the extract, nitrogen excretion through the urine was 0.81 gm. per day below its control level. Since the extracts increase metabolism, the increase in the caloric value, as well as the protein, of the diet may have been a factor in this conversion of a negative result into a positive one. However, during the 2nd and 3rd weeks after injection, the nitrogen excreted in the urine rose 1.08 and 1.37 gm. per day above its control level, and 4 weeks passed before the original level was reached again. During the week from Jan. 15 to 21, a small negative balance of about 0.4 gm. per day undoubtedly existed, since the nitrogen loss in the feces on this diet has usually averaged a gram per day.

is discussed briefly, as is the possibility that the heterogeneity of the x-rays accounts for all or part of the increased bactericidal effect of the rays in the presence of heavy metal salts.

6. Results indicate that within the range investigated, short wave lengths of x-rays, in conjunction with sensitizers, are the more efficacious in producing lethal effects.

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six out of ten experiments on Dog 1. But in respiration experiments recorded in Table IV, Dog 1 was used again, and the daily urine nitrogen values clearly reflect the above tendency. The high level at the start, April 18, was the sequel of a previous treatment, and this high level is reached again during the 2nd or 3rd week after the injections of April 21 and 22, and May 12.

TABLE III

The Effect of Anterior Pituitary Extract on the Urine Nitrogen Partition, Water Intake and Output, and Body Weight

Dog 4. Nitrogen intake, 10.15 gm. daily.

Date	Total N	Ammonia N	Urea N	Creatinine N	Water intake	Urine volume	Body weight	Extract given subcutaneously
1932	gm.	gm.	gm.	gm.	cc.	cc.	kg.	
June 14	8.94	0.28	7.72	0.240	500	370	22.1	50 cc. No. 094891A, boiled*
15	8.74	0.27	7.29	0.243	550	580	22.1	
16	8.84	0.29	7.49	0.227	650	550	22.1	
17	8.34	0.31	7.31	0.229	535	325	22.3	
18	8.68	0.32	7.39	0.226	655	565	22.3	
19	8.96	0.27	7.55	0.229	360	395	22.2	
20	8.48	0.23	7.12	0.231	640	400	22.4	
21	8.54	0.45	7.29	0.234	750	250	22.7	
22	4.86	0.45	3.63	0.238	1280	830	22.8	
23	6.62	0.33	5.55	0.249	1400	1040	22.6	50 cc. No. 094891A
24	7.76	0.32	6.52	0.214	750	700	22.6	
25	8.08	0.29	7.06	0.212	780	710	22.6	
26	8.63	0.29	7.51	0.238	520	510	22.6	
27	8.82	0.28	7.76	0.244	480	560	22.5	
28	9.40	0.30	8.08	0.253	640	565	22.4	
29	9.68	0.32	8.38	0.234	425	465	22.3	
30	10.05	0.32	8.75	0.232	750	590	22.4	
July 1	10.40	0.30	8.95	0.231	440	520	22.3	

* Boiled 5 minutes. Butyl alcohol replaced (see footnote, Table I).

Nitrogen Partition.—The data recorded in Table III were obtained in an experiment on Dog 4. Both the immediate fall in nitrogen excretion, and the rise during the 2nd week after injection of extract, are due to changes in urea excretion. There is a definite increase in ammonia output during the days immediately following the injection. Creatinine excretion, as in Table I, shows no remarkable changes. The diets were not creatine- and creatinine-free, but were constant for

SOME EFFECTS OF ANTERIOR PITUITARY EXTRACTS ON NITROGEN METABOLISM, WATER BALANCE, AND ENERGY METABOLISM

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Prolonged administration of certain anterior pituitary extracts to animals results in at least two processes requiring nitrogen,—growth and lactation. It is obvious that in the production of gigantism in rats, by Evans and his coworkers (1, 2), and in the production of acromegaly in dogs by Putnam, Benedict, and Teel (3), large amounts of nitrogen must have been involved, although no observations on the nitrogen balance were made in the particular experiments. As a result of single injections of anterior pituitary extract, Teel and Watkins (4) observed a fall in the blood non-protein nitrogen of dogs. Teel and Cushing (5) also refer to experiments carried out in collaboration with Dr. Walter Bauer, in which it was found that anterior pituitary extracts administered to dogs which were consuming a constant diet caused a marked retention of nitrogen. The fall in blood non-protein nitrogen observed by Teel and Watkins indicated that this retention was not secondary to renal impairment.

It appeared of interest to the writer to study in some detail the effect of anterior pituitary extracts on the nitrogen economy of dogs. The term "growth" is at once recognized as a collective name for a considerable series of processes, and the term "growth hormone" suggests a single substance capable of initiating all of these. Should injections for a day or two result in a permanent gain in nitrogen, in absence of lactation, this result would be compatible with the idea that all of the processes required for laying down of permanent tissue had been stimulated and had gone to completion. On the other hand, a subsequent loss of the stored nitrogen would suggest that the factor supplied merely stimulated formation of reserve or "deposit" protein,

next 24 hour period. In contrast with the effect on heat production, to be discussed later, the effect on temperature is small and transient.

Lactation.—Marked lactation followed the injections given to Dog 1 on November 23 and 24, 1931, during the experiments recorded in Table I.

About 36 hours after the injections all the breasts, including the poorly developed anterior pair, were very tense. The distal two showed a circular area of decided hyperemia, 5 cm. in diameter, surrounding the nipple. On the following day there were numerous drops of milk on the urine pan, and the cage urine was visibly turbid from it. This continued for 3 days, after which the swelling of the breasts retrogressed. In the course of 10 injections of 50 cc. or more which were given to this animal in the course of a year, this striking result was obtained only once, although after injections given May 12 and June 9, 1932, the breasts were enlarged and milk could be expressed. Eight injections given to the other two dogs never produced lactation, even though in one case the same lot of extract which produced the marked lactation above mentioned was used.

Heat Production and Respiratory Quotient.—In Table IV the action of the extracts on heat production and on the respiratory quotient is shown.

The animal, Dog 1, had been used for respiration experiments for nearly 4 months previously. It received 200 gm. of beef heart at noon, and 200 gm. of ground mixture containing 1 part of beef heart to 2 parts of cracker meal, at 4 p.m., the diet being intentionally arranged to give fairly high basal respiratory quotients. All of the food was consumed within a few minutes after 4 p.m. The metabolism determinations were made between 10 a.m. and 12 noon, after the animal had been connected with the apparatus for a preliminary hour. Injections were given at 9 a.m., hence metabolism determinations recorded on the same day as injections followed these injections by 2 to 3 hours. The figures for calories per hour, and for non-protein respiratory quotients, are the averages of the two 20 minute periods. The basal metabolism falls about 2 calories in the course of the month of experiments. Injections of boiled extract on Apr. 19 and 20 had no effect. When active extracts were given on the 21st and 22nd, a slight rise in heat production was already noticeable on the 21st, 3 hours after the first extract was given. On the next day no experiment could be carried out, due to the marked panting which occurs invariably when extract is given in large doses on 2 successive days, and also due to the fact that depriving the animal of water for 3 hours would produce a temperature rise great enough to complicate the result. However, in a day or two conditions were suitable again for calorimetry. On the 23rd the heat production was found at 42.5 calories per hour, or 77 per cent above its control level. The original value was not reached again until 14 days after the injec-

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400-2
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varied plus or minus 0.5°F . from an average of 100.6°F . The great increase in heat production was accompanied by the usual increases in respiratory rate and ventilation rate, which subsided along with the heat production. The non-protein respiratory quotients on the day of injection (2 to 3 hours after the injection) showed little change, but on the following days there was a marked fall of the R.Q. During this time there was no sugar in the urine. Moreover the fall in R.Q. was due to the oxidation of additional fat, to meet the animal's increased energy requirements, rather than to a decrease in carbohydrate oxidation. For the three control days preceding the first injections, and the two preceding the second, the complete calculation of the experiments showed an average oxidation of 2.98 gm. of carbohydrate, 0.36 gm. of fat, and 1.77 gm. of protein per hour. For the 4 days when R.Q.'s were low, following injections, the average results were: 2.18 gm. of carbohydrate, 2.43 gm. of fat, and 0.97 gm. of protein per hour. With carbohydrate intake constant, protein catabolism reduced, and heat production greatly increased, the available carbohydrate 18 hours after the last meal was no doubt reduced sufficiently to account for the small decrease in oxidation of it.

DISCUSSION

With respect to nitrogen metabolism, the constant finding in these experiments has been that within 24 hours after injection of the anterior pituitary extract into adult dogs kept on a high protein diet, there is a large fall in urine nitrogen, persisting for several days. This result was obtained in each of ten experiments on Dog 1, and four experiments on Dog 4, and also in the case of the younger animal, Dog 2, after it was given a high protein diet. The subsequent result appears to be variable. Although not all of the data are shown, all of the results were of the types presented. There may be a return of urine nitrogen to its former level (Table I) without evidence that the stored nitrogen is lost again, or storage already taking place may be augmented during the week following injections, and correspondingly diminished during the following weeks (Table II), or the stored nitrogen may be lost again (Chart 1). It is remarkable that the nitrogen storage occurs in spite of other effects of the extract,—a slight rise in temperature, a great increase in metabolism, and a large increase in water intake and output, which would all favor nitrogen loss,—and that storage ceases or gives way to loss when these unfavorable conditions have disappeared.

Evidence of interference with carbohydrate metabolism was not even transiently seen in the experiments under consideration. The

the bladder, the volume being measured before washings were added. The urine were collected under toluene, and were analyzed soon after termination of the period. Nitrogen analyses of urine, feces, and food were carried out by the Kjeldahl-Gunning method. Ammonia and urea in urine were determined by the procedure of Van Slyke and Cullen (9), creatinine by the method of Folin and Wu (10). Blood was analyzed for non-protein nitrogen by the method of Folin and Wu (11).

Respiration calorimetry was done by the gasometer method. The procedure was similar to that of Boothby and Sandiford (12), excepting that a plaster of Paris mask, rendered air-tight by a coating of paraffin, was used instead of a copper mask. The animal was trained to cooperate so that the mask could be made without anesthesia, upon its head as a mould. At the time that this was done padding was placed under the lower jaw so that in the eventual mask there would be space enough to permit the animal to breathe over its tongue.¹ A 2.25 inch length of tubing, large enough to take a No. 12 rubber stopper, was incorporated in the front end of the mask. The inlet and outlet tubes passed through the stopper. In making the connection between the rear of the mask and the shaven neck of the animal, 6 inch heavy dental dam was used. A test for leaks was made at the end of each experiment, by applying soap suds while the outlet tube of the mask was dipped under 10 to 15 cm. of water, thus inducing the animal to exhale against pressure. Daily shaving of the animal's neck was found to be unnecessary if a coating of a gel was applied. A suitable gel was prepared by stirring 20 gm. of gum ghatti² into 400 cc. of boiling water, and cooling. This gel does not damage rubber quickly, and washes off readily. Basal metabolism determinations were made during two 20 minute periods at least 18 hours after food. The urine for a 3 to 4 hour period during which these determinations were made was collected and analyzed for nitrogen. The calories per hour and non-protein respiratory quotients were calculated according to the directions given by Lusk (13) and the revisions subsequently published (14).

The pituitary extracts were obtained through the courtesy of Parke, Davis and Co. They were prepared by a method described by Bugbee, Simond, and Grimes (15), in which the solution obtained by alkaline aqueous extraction of the anterior lobes of beef pituitary glands is salted out with sodium sulfate, and the precipitate obtained is redissolved in such a volume of 0.02 N NaOH that 2 cc. of solution equal 1 gm. of anterior lobe. The solution injected contained 1.41 mg. of nitrogen per cc., hence only 0.07 gm. per 50 cc. Creatinine could not be detected. Thus errors in the nitrogen balance or partition are not introduced by injections. Injections were made either intraperitoneally or subcutaneously. In the latter instance the 50 cc. doses were injected in 5 or

¹ For the information that this condition should be met, the writer is indebted to Dr. Boothby.

² Preparations vary. The powdered preparation of J. L. Hopkins and Co., New York, was used.

stimulating fraction of the pituitary exerts any similar action on the nitrogen economy will be investigated.

CONCLUSIONS

1. Solutions of the globulin fraction of alkaline extracts of the anterior lobe of beef pituitary glands, when administered intraperitoneally or subcutaneously to dogs for 1 or 2 days, greatly increase the nitrogen balance. Urine nitrogen falls markedly, and at the same time blood non-protein nitrogen decreases, while the nitrogen of the feces remains the same.

2. The nitrogen gained may either be retained or lost again, indicating that it may be converted into reserve protein rather than into permanent structures. Sudden gains in weight which follow the injections are always lost again.

3. The effect of the extracts on protein catabolism is greatest when this is high, as in adult dogs having a negligible positive nitrogen balance, or a negative balance, while consuming a high protein diet adequate in calories.

4. Both the fall in nitrogen excretion, and the subsequent rise, when the latter occurs, are due to changes in urea excretion.

5. Other results of the brief treatments are: great increases in water intake and urine volume, marked thirst, slight rise in temperature, and a remarkable increase in heat production effected by oxidation of fat. Mature female dogs were used, and in one of these lactation occurred after some of the injections.

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TABLE I
Effects of Anterior Pituitary Extracts on Nitrogen Balance and Urine Volume
 Dog 1. Nitrogen intake, 10.11 gm. daily.

Date	Urine analysis			Blood non-protein N mg./100 cc.	Feces total N gm.	Extract given subcutaneously
	Total N gm.	Creatinine mg.	Volume cc.			
1931						
Nov. 5	8.85	590	660			
6	9.13	610	750			
7	9.11	608	480			
8	9.14	600	630			
9	9.81	610	520			
10	8.69	587	750			
11	8.96	592	525			50 cc. No. 094828A* 50 cc. No. 094891A*
Average...	9.10	600	616			
Nov. 12	9.62	615	530			
13	4.73	550	1090			
14	5.24	610	1545			
15	7.23	608	1640			
16	7.25	577	805			50 cc. No. 094891A 50 cc. No. 094891A
17	7.92	595	660			
18	9.06	605	780			
19	8.88	590	635			
20	9.18	595	680			
21	9.40	595	580			
22	9.15	598	585	47.0 47.6		
Average...	7.97	594	866	1.14		
Nov. 23	8.92	625	510			
24	4.72	635	1460	30.9		
25	3.97	613	1740	30.7		
26	6.16	663	1450	35.5		
27	8.00	605	1245	37.2		50 cc. No. 094891A 50 cc. No. 094891A
28	8.23	577	675	37.5		
29	8.70	624	620	37.6		
30	8.72	606	1015	37.0		
Dec. 1	9.51	595	590	42.8		
2	9.03	625	615	38.0		
Average...	7.60	617	992	1.14		
Dec. 3	9.42	604	680			
4	9.07	594	650			
5	9.05	600	680			
6	9.27	592	900			
7	8.73	595	740			25 cc. tomato juice, 10 cc. cod liver oil added to daily diet
Average...	9.10	597	730			

* Extract boiled 10 minutes over a screen; 1 cc. of butyl alcohol then added, since the extracts are preserved with 2 per cent of butyl alcohol, some of which is lost during boiling.

In Chart 1 is presented an extreme instance of the tendency for a period of nitrogen storage as a result of injections of anterior pituitary extract to be followed by a period of nitrogen loss.

The animal, Dog 4, weighed 22.1 kilos, and received 200 gm. of beef heart and 200 gm. of cracker meal-beef heart mixture per day. The nitrogen intake was 10.35 gm. per day. The marked fall in urine nitrogen as the result of extract injections on Jan. 1 and 2 is shown in the chart. But soon afterwards the nitrogen of

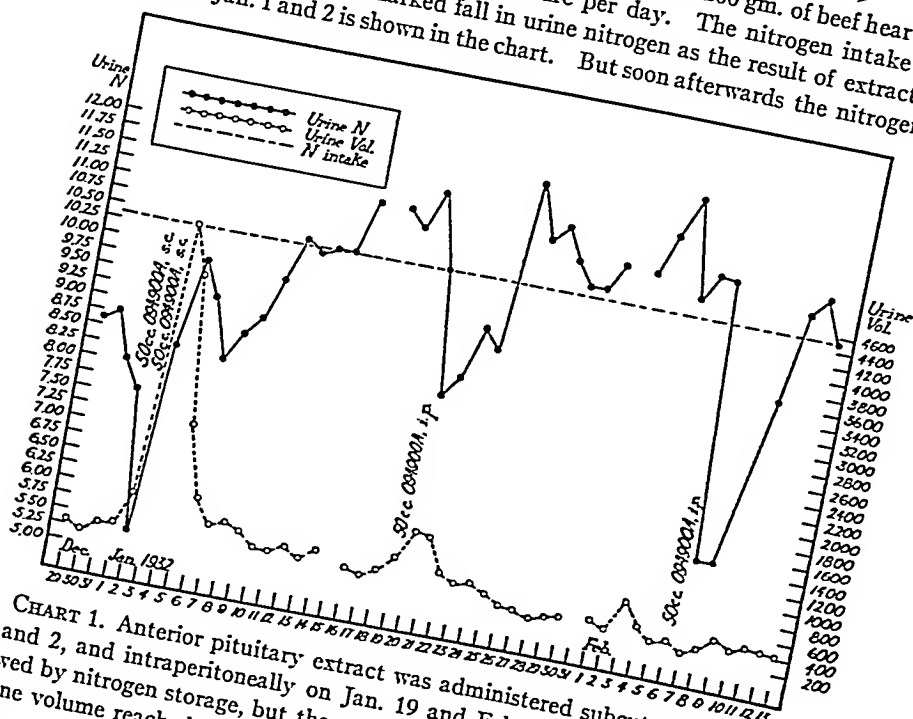


CHART 1. Anterior pituitary extract was administered subcutaneously on Jan. 1 and 2, and intraperitoneally on Jan. 19 and Feb. 6. Each injection was followed by nitrogen storage, but the stored nitrogen was lost again. On Jan. 3 the urine volume reached 4490 cc.

the urine alone was greater than the nitrogen intake. At this high level of protein catabolism further injections, in this case given intraperitoneally, had a striking action, and restored the positive nitrogen balance temporarily, but the animal always returned to the negative balance. In the period following that shown in the chart the urine nitrogen very slowly returned to a level about 1 gm. below the intake. The period of irregular nitrogen losses shown in the chart was unaccompanied by fever, and the animal had a good appetite and was in excellent condition.

The tendency for nitrogen storage to give way to nitrogen loss was not observed in the experiment recorded in Table I, nor, in fact, in

Table I demonstrates the wide variation between different experimental groups in the characteristics analyzed. For instance, the incidence of the disease as measured by percentage microscopic lesions, is seen to vary from 30 to 100 per cent. Of the total number, about one-third developed neither symptoms nor brain lesions during the arbitrarily chosen experimental period. Of the 99 chicks which showed symptoms, 83 presented lesions which were noted both grossly

TABLE I
Incidence of Nutritional Encephalomalacia in Chicks on Diet 108

Date of hatch	Length of time on stock diet	No. of chicks	No. of chicks with			Per cent with microscopic lesions	Length of time until end of experiment	Mean No. of days until onset of disease
			Symptoms	Gross lesions	Microscopic lesions			
	<i>days</i>						<i>days</i>	<i>days</i>
June 8, 1930	10	10	6	3	5	50	40	20
July 7, 1930	15	7	7	6	7	100	28	20
Sept. 30, 1930	7	22	8	10	13	54	28	20
Nov. 14, 1930	10	11	5	5	6	54	30	24
Jan. 8, 1931	2	11	7	7	7	64	28	22
Feb. 26, 1931	0	15	10	7	7	47	28	22
Apr. 30, 1931	0	20	9	5	6	30	35	18
Sept. 28, 1931	0	13	9	7	8	62	32	26
Dec. 1, 1931	0	13	7	8	9	69	42	36
Mar. 1, 1932	0	18	8	8	10	56	46	26
Apr. 26, 1932	0	15	12	12	13	87	56	34
June 13, 1932	0	17	11	7	7	41	42	28
Totals		172	99	85	98	57		

and microscopically; 12 showed microscopic lesions only, and 2, gross; in 13, no lesions were found. The presence of symptoms without demonstrable lesions may have been due to the fact that it was impracticable to cut serial sections of the entire brain in each case, and that small microscopic lesions may have escaped recognition. Another possible explanation is that the symptoms were due to transient functional disturbance—perhaps vascular spasm—not followed by manifest anatomical changes in the brain tissue.

The variation between groups may be due in part to the fact that

months. A nitrogen partition experiment on Dog 1 yielded results completely in agreement with those in Table III. Allantoin excretion was not studied. The immediate increase of ammonia excretion is unexplained. It precedes the rise in water intake, which might otherwise be considered the cause (16). Moreover the extracts injected are not acid, but alkaline.

Water Balance and Body Weight.—In Table I the marked increase in urine volume after injections of active extract is evident at once. Boiled extract does not have this action. In Chart 1 the injection of 100 cc. of extract over a 2 day period, January 1 and 2, is shown to increase the urine volume to 4490 cc. per day. The animals, after such injections, have an amazing thirst. The 50 cc. intraperitoneal injection given on February 6 (Chart 1) had little effect on urine volume, and this is often the case, although the effect on the nitrogen balance is as great as in the case of subcutaneous administration. 50 cc. doses given subcutaneously as a rule increased the urine volume to about 1100 cc.

In Tables III and IV the water intake (not including approximately 315 cc. of water contained in, or obtainable by oxidation of, the 400 gm. of food given daily) is shown along with the urine volume and body weight. The water intake increases even more than the urine volume, and there is an abrupt rise of 300 to 500 gm. in body weight when active extracts are given. In four such experiments, three of which are shown in Tables III and IV, the weight gain was always lost again. Table IV has been shortened, since other data were of no interest, but the weight proceeded downward daily to 20.9 kilos on May 24, and then remained constant. Since injections, urine data, and body weights appear opposite the same date, it should be stated that the injections were given at 9 a.m. on the day indicated; the urine data are for the 24 hour period beginning with the injection, and the weight was determined at the end of this period, after catheterization.

Body Temperature.—In three experiments daily rectal temperatures were taken in the morning, and at intervals of 3 to 4 hours on days when 50 cc. injections of extract were given. As long as the animals had free access to water the rise in temperature was only 1°F. at its height, 0.5°F. 24 hours after injections, and disappeared during the

The Influence of Age upon Susceptibility

The experiments summarized in Table I were carried out upon day old or very young chicks; our few negative results with older birds had given the impression that this disorder could be induced only during the early growth period. In order to obtain more definite information upon this point, groups of chicks of the same hatch were placed upon the disease-producing Diet 108 after varying intervals upon the natural foods Diet 634 of Hogan, Hunter, and Kempster (4).² Those that showed no symptoms were kept under observation for at least 40 days.

The incidence of the disease in these successive groups and the time elapsing until the onset are shown in Table II.

From the results of this experiment it would appear that the susceptibility to the disease diminishes as the preliminary period upon the natural foods diet is extended. It might be supposed that the older chicks would exhibit the disease in a milder form and that it would take longer to develop. This is definitely not the case. Thus in Chick 1294, which had been on the natural foods diet for 54 days, and which was the only one of 8 in this group to show the disease, severe symptoms appeared suddenly after 10 days on Diet 108. It was immediately killed and extensive fresh lesions were found in the cerebellum.

Table II demonstrates the unexpected fact that the disease tends to develop after a shorter period in the older chicks, in spite of the fact that fewer of them become affected.

Growth and Susceptibility

For purposes of comparison with the growth of chicks on Diet 108, a

² Diet 634 of Hogan, Hunter, and Kempster:	per cent
Whole wheat.	55.6
Whole milk powder.	8.2
Casein.	12.3
Alfalfa meal.	2.5
Butter fat.	4.2
NaCl.	0.9
CaCO ₃	1.3
Cod liver oil.	3.0
Yeast.	12.0

tions. A single dose of 50 cc. of extract given on May 12 also produced a definite rise within 3 hours, reaching a maximum of +68 per cent on the following day, and

TABLE IV
Changes in Energy Metabolism, Weight, and Water Balance after Injections of Anterior Pituitary Extracts

Dog 1. Nitrogen intake, 10.40 gm. daily.

Date	Urine N	Water intake	Urine volume	Body weight	Calories per hr.	Non-protein R.Q.	Extract given subcutaneously
	gm.	cc.	cc.	kg.			
1932							
Apr. 18	10.65		600	21.0	23.6	0.94	
19	10.83	390	350	21.0	24.3	0.89	
20	9.62	470	260	21.1	23.9	0.90	50 cc. No. 095052A boiled*
21	9.12	770	330	21.6	25.3	0.94	50 cc. No. 095052A boiled*
22	6.48	1140	950	21.6	42.5	0.78	50 cc. No. 095052A
23	5.99	2500	2425	21.5	35.1	0.79	50 cc. No. 095052A
24	8.48	2130	1900	21.6	28.6	0.93	
25	8.98	1375	1160	21.1	25.4	0.89	
May 3				21.0	23.7	0.90	
4				20.9	23.1	0.90	
6	9.96	570	515	20.9	21.8	0.99	
7	10.07	565	530	20.9	21.2	0.97	
8	10.38	475	475	20.9	25.4	0.97	
9	10.43	600	570	20.9	36.7	0.79	
10	9.77	595	700	21.2	31.8	0.78	
11	10.19	600	315	21.3			
12	10.19	600	750	21.2			
13	5.39	1040	1195	21.2			
14	6.13	1360					
16	8.53	1280	520	21.5			
17	8.90	790	655	21.5			
19	10.11	670	680	21.2			
20	10.63	820	700	21.2			

* Boiled 5 minutes. Butyl alcohol replaced (see footnote, Table I).

requiring 7 to 8 days to subside completely. During the experiment on Apr. 23 the rectal temperature was 1.4°F. above its average, and during the experiment on May 12 0.9°F. above this average. On all other days the rectal temperature

lacia that survived the diet for more than 28 days before showing symptoms. Curve C refers to 30 chicks in which the brain was normal at autopsy. Curves B and C are seen to be similar. The growth is not nearly so good as that for chicks on the natural foods Diet 634.

The records of many chicks that did not survive even 21 days were available. The average daily increase in weight of these is included in the data given in Table III.

TABLE III
Mean Daily Increase in Weight of Chicks on Diets 108 and 634

Diet	No. of chicks	Mean daily increase in weight	P. E. of mean	Standard deviation	P. E.	Coefficient of variation
		gm.	gm.	gm.	gm.	gm.
108 (with lesions)	67	2.9	0.08	0.9	0.05	31.6
108 (without lesions)	56	2.8	0.09	1.0	0.06	36.9
634 (normal chicks)	85	6.2	0.11	1.5	0.08	24.9

TABLE IV
Incidence of Nutritional Encephalomalacia in Different Breeds of Chicks on Diet 108
Chicks Hatched March 1, 1932

Breed	No. of chicks	No. of chicks with			Per cent with microscopic lesions	Mean No. of days until onset of disease
		Symptoms	Gross lesions	Microscopic lesions		
White Leghorn	18	9	11	10	56	26.1
Barred Plymouth Rock	16	11	11	10	62	28.7
White Wyndotte	20	15	12	12	60	28.5
Rhode Island Red	18	11	14	15	83	29.2

From Table III it is apparent that chicks on Diet 108 had the same mean daily increase in weight irrespective of whether or not they developed the disorder.

The Susceptibility of Different Breeds to Nutritional Encephalomalacia

In the following experiment, the susceptibility of White Leghorns, Barred Plymouth Rocks, Rhode Island Reds, and White Wyandottes

urine of all animals was frequently tested for sugar with negative results, and respiration experiments on the animal which was treated longest indicated a ready oxidation of carbohydrate. Hyperglycemia and glycosuria in dogs is a very late result of treatment with pituitary extracts. Putnam, Benedict, and Teel found no glycosuria or hyperglycemia in their injected animal after 3 months of daily injections, and no hyperglycemia after 8 months. Evans, Meyer, Simpson, and Reichert (17) found hyperglycemia and glycosuria in two of four dogs after daily injections for 8 to 9 months. In rabbits, marked hyperglycemia and glycosuria were produced within 1 or 2 weeks by Baumann and Marine (18), who injected saline extracts of pituitary tissue. The marked increases in water intake and output found in the present experiments are confirmatory of findings already well known (5). The sudden increase in weight, indicating increased hydration, is soon lost again.

With regard to heat production, the marked increase observed after injections in these experiments is similar to the effect of alkaline extracts and other pituitary preparations on the metabolism of guinea pigs (19). The writer had hoped to make observations on the specific dynamic action of protein during the period following injections of extract, when the catabolism of protein given is reduced by one-half. But during this period of a few days after injections one is fortunate if enough reliable observations to establish the basal metabolism are obtained, and the animals are not suitable for experiments lasting many hours.

For biological assays, the ideal procedure is one in which a large change is produced in a short time by a single injection. It was partly for this reason that the effects of injections for only 1 or 2 days were studied. But as has been indicated above, some of the effects of the extracts are antagonistic to others, and accurate standardization on the basis of the effect on nitrogen excretion is therefore unlikely at present. The effect on nitrogen excretion is, however, a fairly simple test to be used in purification of the substance responsible for it, and in this connection the conditions under which a marked response is obtained in a short time are of interest. Such a substance could of course be involved in both growth and lactation. Whether the gonad-

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formula weight. These substances, partial hydrolysis products of the specific polysaccharide of Type III pneumococcus, precipitate Type III antipneumococcus horse serum and parallel in formula weight the series of hapten dyes recently described by Landsteiner and van der Scheer (8) as precipitating rabbit antisera.

A marked qualitative difference between antibodies to Type III pneumococcus engendered by the horse and those produced by the rabbit is brought to light by the failure of the partial hydrolysis products of S III¹ to precipitate the antibody in rabbit serum. Other differences of a quantitative nature will be discussed in a subsequent communication.

EXPERIMENTAL

Hydrolysis of S III with 70 per cent sulfuric acid and the separation of the hydrolysis products into fractions of different average molecular weight have been described in previous papers (9, 7). Unless the conditions are rigorously controlled, very stable sulfuric acid esters of the hydrolysis products may be formed. To eliminate this difficulty an additional series of fractions was prepared using hydrochloric acid as the hydrolytic agent.

The S III was suspended in 1:1 hydrochloric acid and hydrochloric acid gas was passed into the mixture until a clear solution resulted, keeping the temperature below 0°. The use of 1:1 acid as the initial solvent resulted in an even suspension of the S III and avoided the jelly-like lumps that formed when concentrated acid was used. The solution was allowed to stand in the ice box overnight. To guard against further hydrolysis of the higher fractions during the removal of the acid, they were precipitated by adding five volumes of 95 per cent alcohol and washed free from hydrochloric acid with alcohol before conversion into the barium salts. The supernatant and washings containing the lower fractions were concentrated to dryness *in vacuo*, keeping the temperature below 35°C. The residue was taken up in water, neutralized with barium hydroxide, and the barium salts were precipitated with alcohol and the process was repeated until the sugar salts were free from chlorides.

Any unhydrolyzed S III still present was removed as the copper salt by adding an excess of 5 per cent copper sulfate solution to a solution of the barium salts of the fractions, keeping the reaction neutral to litmus with barium hydroxide. After standing overnight the precipitate was centrifuged off and washed with water containing a little copper sulfate. The supernatant and washings were acidified with sulfuric acid and the copper was precipitated with hydrogen sulfide.

¹ This abbreviation is used to designate the specific polysaccharide of Type III pneumococcus.

NUTRITIONAL ENCEPHALOMALACIA IN CHICKS*

INFLUENCE OF AGE, GROWTH, AND BREED UPON SUSCEPTIBILITY
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In previous papers (1, 2), attention has been called to a nutritional disorder of young chicks, characterized by severe injury to the central nervous system. During the past 2 years, experimental studies have been continued in the as yet unfulfilled hope of defining the precise factors responsible. The data which have been accumulating enable us to discuss in the present paper, the influence of age, growth, and breed upon the occurrence of this interesting disorder.

The behavior of 172 White Leghorn chicks, belonging to 12 groups that were placed upon the disease-producing Diet 108¹ either at hatching, or after a short preliminary period on a natural foods diet, has been summarized in Table I with respect to the percentage incidence, the occurrence of symptoms, gross and microscopic lesions, and time elapsing until the appearance of the disease. Chicks that died during the 1st week or that had doubtful lesions, are not included in the table.

* This work was aided by the Research Grant from the Chemical Foundation to the Department of Biological Chemistry.

¹ Diet 108 has the following composition:

	per cent
Skimmed milk powder (Merrell-Soule).....	15.0
Cascein (Merck's technical).....	20.5
Cornstarch.....	20.0
Lard.....	21.0
Cod liver oil (Mead Johnson and Co.).....	2.0
Yeast (Fleischmann's bakers', dried).....	5.0
Salt mixture (McCollum 185) (3).....	6.5
Paper pulp (Eastman).....	10.0

portions of the dilutions of the fractions and 0.5 cc. portions of an antibody solution prepared from Type III antipneumococcus horse serum. The antibody solution contained 5.0 mg. of specifically precipitable protein per cc. Readings were taken immediately after mixing and after 2 hours in the water bath at 37° and overnight in the ice box. Corresponding tests with Type III antipneumococcus rabbit serum or antibody solution were negative, even after centrifugation.

DISCUSSION

It was originally reported that the partial hydrolysis products of S III did not precipitate Type III antipneumococcus serum (9). It has now been found that, even at a dilution of 1:1,000,000, all of the fractions except the aldobionic acid precipitate the homologous horse antiserum. The reaction differs from that of the unhydrolyzed S III in that the precipitating zone is narrower, chiefly owing to the inhibiting action of higher concentrations, as shown in Table II. Inhibition of precipitation, as read immediately after mixing, results in the concentrations used for testing in the previous studies, while under the normal conditions for the precipitin test a wider precipitating zone is found.

The possibility that the precipitate obtained is due to traces of unhydrolyzed S III in the fractions is excluded by several considerations. In the first place, unhydrolyzed S III is completely precipitated, even from very dilute solutions, by neutral copper sulfate. The supernatant from a copper-treated 1:20,000 dilution of S III contains less than 1:10,000,000 S III as shown by comparative precipitin tests. The purified hydrolytic fractions are not precipitated by copper salts under these conditions and even in high concentration do not inhibit the copper precipitation of added S III.

In the second place, quantitative determinations show that the amount of antibody precipitable by the fractions is much greater than could be caused by contamination with unhydrolyzed S III. The last two columns in Table I show the amounts of nitrogen precipitated from an antibody solution by 0.05 and 0.15 mg. of the different fractions and by S III for comparison. It will be seen that the hydrolysis fractions of higher molecular weight precipitate practically as much antibody as does an equal weight of S III. Moreover, a quantitative study of the entire range of precipitation² shows that the reaction in the case of the fractions differs from that of S III with antibody,

² To be reported in another communication.

chicks were not allowed to survive for a sufficiently long period. This possibility was not fully appreciated in the earlier experiments, in which all the survivors—*i.e.* those which had not manifested symptoms—were killed after an arbitrary period on the diet, the length of which is listed in Table I. Recently, we have observed a chick in which the disease appeared suddenly after 53 days, and a number of others in which it first appeared after 40 days. In a recent group of 25 chicks, 11 developed the disease before the 28th day and 14 afterwards.

TABLE II
Incidence of Nutritional Encephalomalacia in Chicks That Were Placed on Diet 108 at Different Ages
Chicks Hatched April 26, 1932

Group No.	Length of time on stock diet	No. of chicks	No. of chicks with			Per cent with microscopic lesions	Mean No. of days until onset of disease
			Symptoms	Gross lesions	Microscopic lesions		
1	0	15					
2	12	15	13	13	13	87	34
3	19	14	13	11	14	93	31
4	26	15	9	8	9	62	23
5	40	16	5	4	6	40	19
6	54	8	3	3	4	25	21
7	68	7	2	1	1	14	10
			0	0	0	0	

The period elapsing until the onset of the disease has been roughly determined by killing the chick upon the first appearance of symptoms and establishing the diagnosis of encephalomalacia from the presence of characteristic lesions. In a certain number of chicks which were found dead or which were killed at the end of the experiment, no clinical abnormalities had been noted, but since these chicks presented fresh lesions at autopsy, they are included in Table I. Not included, however, are chicks showing symptoms but no lesions, or only old healed or healing lesions.

Table I shows that the variation is not dependent upon seasonal influences. Thus in April, 1931, only 30 per cent showed the disease, as against 87 per cent in the same month of 1932.

chiefly in the greater inhibiting effect of increasing concentrations. This is also shown qualitatively in Table II.

Finally, rabbit antisera that precipitate heavily with unhydrolyzed S III are not precipitated by any dilution of the fractions, even when the concentration of protein specifically precipitated by S III is as high as 17 mg. per cc. of serum. Combination of the fractions with rabbit antibody occurs, nevertheless, to form soluble compounds, since relatively high concentrations of the fractions inhibit specific precipitation with S III. Felton has shown that antibodies to *Pneumococcus* in horse sera are precipitated with the water-insoluble fraction of the serum globulins (12). Rabbit antipneumococcus sera, however, yield no precipitate on dilution, so that it is possible that the failure of the hapten fractions to form insoluble compounds with rabbit antibody may be connected with the greater tendency of rabbit globulin to remain in solution. This tendency, however, does not prevent even as dilute a solution of S III as 1:10,000,000 from precipitating rabbit antibody.

The difference between rabbit and horse antibodies to *Pneumococcus* as regards their ability to precipitate the S III fractions raises the question whether or not other haptens of low molecular weight might more often prove to be precipitating as well as inhibiting were immune horse sera used instead of the rabbit sera now almost universally employed. Thus Landsteiner and van der Scheer have observed innumerable inhibition reactions with simple haptens, but reported positive precipitin tests only in the case of the azo dyes formed by coupling *p*-amino-succinanilic acid and its homologs with resorcinol or tyrosine (8).

SUMMARY

1. Partial hydrolysis products of the specific polysaccharide of Type III pneumococcus ranging from 550 to 1,800 in formula weight can be quantitatively freed from unhydrolyzed polysaccharide.

2. The fractions yield specific precipitates with Type III antipneumococcus horse serum but fail to precipitate homologous rabbit antisera, giving rise only to specific inhibition. The aldobionic acid, the structural unit of S III, does not precipitate antisera.

3. A possible explanation and a possible application of the findings are pointed out.

composite growth curve A in Chart 2, was constructed of 78 chicks on the Hogan, Hunter, and Kempster natural foods Diet 634 (4). The standard deviations for the initial weight and the weights at 21 and 28 days respectively were calculated in addition to the mean weights and plotted in Chart 1 in order to illustrate the extent of

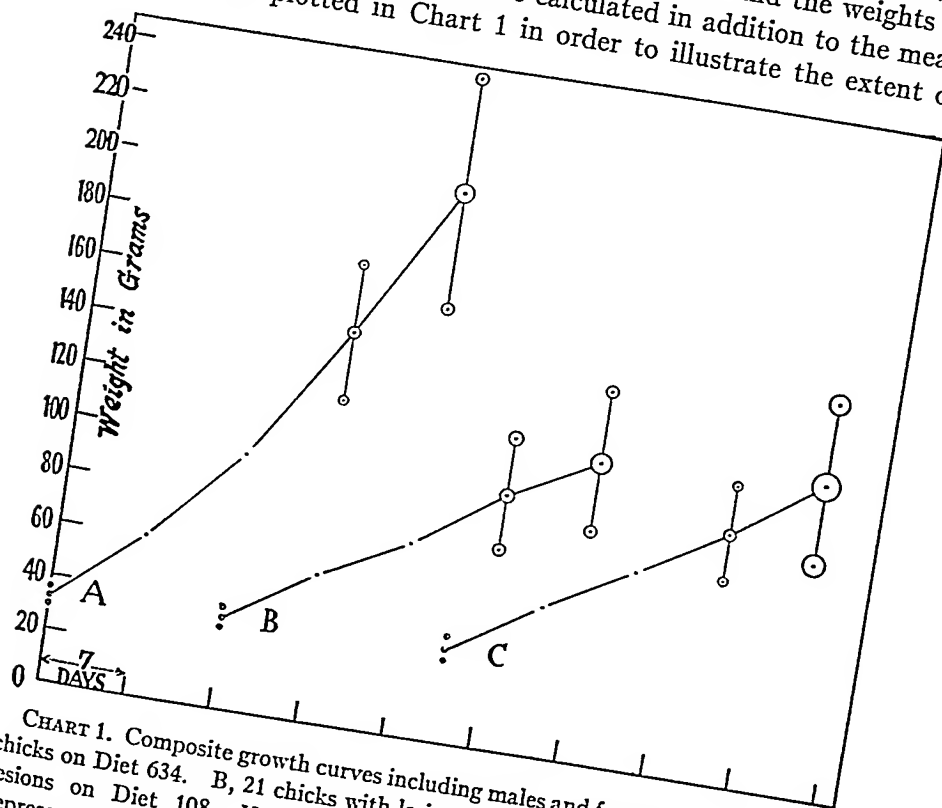


CHART 1. Composite growth curves including males and females. A, 78 normal chicks on Diet 634. B, 21 chicks with lesions on Diet 108. C, 30 chicks without lesions on Diet 108. Vertical lines represent standard deviations. Circles represent probable errors.

biological variation. The probable errors of the mean and of the standard deviation have been depicted by circles. The rate of growth of these chicks on Diet 634 compares favorably with that cited by Hogan, Hunter, and Kempster in their description of the diet, and with that obtained by Mitchell, Card, and Hamilton (5) in a recent study of growth in the White Leghorn chick. The growth curves of chicks on Diet 108 were plotted in a similar fashion. Curve B was constructed from 21 chicks with encephaloma-

was compared. Twenty chicks of each breed were placed on Diet 108 from the day of hatching; 5 additional chicks of each lot were given the natural foods Diet 634. It was found, as shown in Table IV, that the disease is readily produced in each of these breeds. The differences in percentage incidence are probably without significance, in view of the variations noted in the White Leghorn series (Table I).

Not only was there a high incidence in all groups, but the average time on the diet before the appearance of symptoms was virtually the same. The character and distribution of the lesions showed the customary variations in all the groups. One may conclude, therefore, that none of the breeds tested showed a peculiar susceptibility or resistance to the disease, and all are equally suitable for experimental purposes.

CONCLUSIONS

1. Nutritional encephalomalacia may be induced in chicks up to the age of approximately 2 months. As the preliminary feeding period on a natural foods diet is increased, the percentage incidence of the disease becomes progressively less. The average time between institution of diet and appearance of the disease tends to diminish.

2. There is no correlation between growth and incidence of the disease.

3. White Leghorns, Barred Plymouth Rocks, Rhode Island Reds, and White Wyandottes are equally susceptible.

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For active immunization in typhus fever we have at the present time a limited choice of possibilities. In an earlier paper with Batchelder one of us has shown that effective active immunization can be carried out with mixtures of convalescent serum and living virus—a method too perilous to be justified for use in man if there is any possibility of accomplishing the same purpose with dead materials. For immunization against the European disease, the carbolized louse vaccines of Weigl (2) seem to have been effective, but it is obvious that the technical difficulties involved in the use of insects considerably complicate practical application. The same objection applies to the recent method described by Dyer and his collaborators (3) in which a vaccine against the endemic typhus was produced from fleas infected with the virus. That immunization is possible by the method originally described by us has been confirmed by Kemp (4), who, however, found that the immunity was not lasting and that the vaccine retained its potency for a short time only. Dyer and his collaborators cite this paper in criticism of our method without, however, considering that Kemp's experiments were carried out with the original, unimproved technique described by us, in which the vaccination material contained relatively few *Rickettsiae* as compared with our later methods. Moreover, the results reported by Dyer in his experiments were in no respect better than those obtained in some of our series, a fact recognized by Dyer, who believes that a better vaccine than he has so far obtained could be prepared eventually by the flea method.

Since the experiments of Weigl, as well as our own, seem to have demonstrated conclusively that immunity in these diseases can be obtained with dead virus, the ideal method would be one in which sufficient amounts of the *Rickettsiae* could be obtained either by direct cultivation or by tissue culture. Direct cultivation has not so far been possible and tissue culture, though easily accomplished, has not as yet yielded adequate amounts of the organisms. We believe that the easiest procedure so far available by which a sufficiently large amount of vaccine can be produced with the Mexican material is the X-rayed rat method described by us. Under conditions of reasonable success, about 15 cc. of a suspension of washed *Rickettsiae*—almost comparable to typhoid vaccine in the number of organisms—can be obtained from a single rat.

STUDIES ON THE PRECIPITIN REACTION

PRECIPITATING HAPTENS; SPECIES DIFFERENCES IN ANTIBODIES*

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(Received for publication, October 19, 1932)

The term "hapten" was applied by Landsteiner (1) to the portion of a complex antigen which determines its specific reactivity rather than the ability to function as an antigen. Thus in a complex azo protein the component attached to the protein through the azo group determines the specificity, while the protein molecule itself enables the complex to function as an antigen in the production of antibodies (2). Landsteiner demonstrated the specificity of the azo component (hapten) by its ability, when present in excess, to inhibit the specific precipitation of the antigen-antibody complex, and considered the inhibiting action to be due to actual combination of the hapten with the antibody.

It is now clear that the specific polysaccharides constitute a distinctive type of hapten which still retains the power to precipitate antibody specifically (3), combining chemically with the antibody in the precipitate and in the inhibition zone (4), just as Marrack and Smith (5) have recently found in the case of the inhibition reaction due to an azo compound.

It was originally assumed that the ability of specific polysaccharides to precipitate homologous antibodies was a function of a relatively high molecular weight (6). However, the writers have shown that the formula weights of the above mentioned specific carbohydrates are probably less than 10,000 (7). In the present communication there is described a series of precipitating haptens ranging from 550 to 1,800 in

* The work reported in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital.

Of the vaccinated pigs, one animal seemed to be completely protected, no swelling occurring at any time, and the temperature remaining at or below 103°, except on the 6th day, when it touched 104° in the afternoon. The subcutaneously vaccinated guinea pig developed no swelling until the 6th day, when the temperature touched 105°. The temperature remained at 104° only 2 days, then promptly dropped to normal. The third vaccinated animal, though running a typical temperature, did not develop swelling until the 6th day, and ran a course much less violent than the control that had been allowed to survive.

There was thus distinctly less severity of the disease in two of the vaccinated guinea pigs, and apparently complete protection in one of them. This protected animal, however, is the only one of the series to which we would attach much importance, since the milder nature of the disease in the others might well have been due to the greater weight of the vaccinated animals. We omit charts, as unnecessary for the description of these experiments.

Experiment II. July 9, 1932. Vaccinated Animals Inoculated with Mexican Typhus Blood.—Since we realized at the time that we were doing Experiment I that we were giving an enormous amount of virus, and since we had bled and defibrinated the blood of the two guinea pigs that had furnished the tunics, we carried out, on the same day, another experiment in which we inoculated intraperitoneally two controls, two intraperitoneally vaccinated animals and one which had been subcutaneously vaccinated with 1 cc. of the mixed, defibrinated blood specimens of the Mexican typhus Guinea Pigs 1 and 2, described in Experiment I. Again there was, unfortunately, a considerable discrepancy in weight between vaccinated animals and controls, the vaccinated guinea pigs all weighing between 680 and 750 gm. each, while the controls weighed 550 and 650 gm., respectively.

In this experiment, as was to be expected, the controls did not develop typhus until much later than they would have had they been inoculated with tunica material. The temperatures did not rise above 104° in the controls until the 8th day and the 11th day, respectively. Swelling did not appear until the 11th and the 12th days, respectively, but was then typical, and *Rickettsiae* were found on the 13th day in one of these animals, killed for transfer. Both of them, in other words, ran late but typical Mexican typhus fever. The three vaccinated animals developed no swelling whatever, though they were observed for 15 days. One of them—and, perhaps significantly, the subcutaneously vaccinated one—never reached a temperature higher than 103°. One of the intraperitoneally vaccinated animals touched 104° for 1 day, the 14th; the other reached this temperature on the 8th and 12th days, respectively, promptly returning to normal in each case. In none of the vaccinated guinea pigs was there any evidence of typhus fever except the slight, temporary temperature rises mentioned above. In none of them did scrotal swelling develop.

After removing the excess of this gas by distillation *in vacuo*, the sulfuric acid was removed and the hydrolyzed S III converted into the barium salts by neutralizing with barium hydroxide. A portion of the solution was tested with copper chloride for traces of S III and the copper precipitation was repeated if necessary. It is possible that precipitation with copper salts removes, besides unchanged S III, higher hydrolytic products than those described in the present paper. Since it was necessary to remove all traces of S III the copper-precipitable material was discarded.

The barium salts were fractionally precipitated with alcohol after concentration of the solution *in vacuo* to a volume at which the barium salts began to separate. Addition of a little water yielded a clear solution. As alcohol was added, only slight precipitation occurred until a concentration of about 10 per cent of alcohol was reached, when considerable material was thrown down. This was centrifuged off and more alcohol was added. Again there was very little precipitation until at a concentration of about 25 per cent of alcohol a second large fraction was precipitated. After this had been removed the remaining barium salts were rendered insoluble by addition of five volumes of alcohol.

The behavior upon the addition of alcohol indicated that each fraction was not entirely homogeneous with regard to molecular weight but consisted of fragments of S III hydrolyzed approximately to the same extent and contaminated with relatively small amounts of material of higher and lower molecular weight. In the sulfuric acid series fractions B, C, and D were precipitated by 10, 25, and 80 per cent of alcohol respectively. In the hydrochloric acid series, in which the hydrolysis was less severe and the lower hydrolysis products were not formed, the corresponding fractions C and D have a larger average molecular weight. The precipitated fractions were washed with alcohol, filtered off, and dried *in vacuo* at 60°.

The aldobionic acid of S III (9) was prepared by allowing a solution of S III in concentrated hydrochloric acid to stand at room temperature for 2 days. After concentration *in vacuo* the barium aldobionate was isolated and converted into the brucine salt, which crystallized from a concentrated aqueous solution. After recrystallization it was reconverted into the barium salt.

The reducing power of the fractions was determined by the Willstätter-Schudel (10) and the Shaffer-Hartmann (11) methods, and the average molecular weight calculated from the mean of the two determinations, assuming one reducing group per molecule. A summary of the properties of the fractions is given in Table I. It must again be emphasized that each fraction is probably not a definite chemical individual, but represents a mixture of substances with not widely different molecular weights, the value given in each case being the average of the mixture. Fraction D in the sulfuric acid series corresponds roughly to a dialdobionic acid.

Solutions of the sodium salts of the fractions for use in the precipitin tests were made by treating solutions of the barium salts with a slight excess of sodium sulfate. The precipitin tests recorded in Table II were carried out with 0.5 cc.

that were lighter than the vaccinated animals. We therefore procured a number of control animals that corresponded in weight more closely to the vaccinated guinea pigs.

The source of material for the experiment done on July 29 was tunica material from a Mexican typhus guinea pig killed on the 6th day, with typical swelling and a moderate number of *Rickettsiae* in the tunica. Each animal in the experiment was injected intraperitoneally with what amounted to approximately a fifth of a tunica, which should represent several hundred times the minimal infectious dose, although the irregularity of the distribution and amounts of *Rickettsiae* in individual tunicas is such that it would be absurd to attempt definite statements in this regard. In the past, we have obtained infection with 1/1000th of a tunica of the ordinary passage animal.

The animals were injected as follows:

Control 1	weight, ^{gm.} 750
" 2	" 680
" 3	" 720
" 4	" 520

(Control 4 was added for the sake of passing the strain, and giving us some idea as to the difference in susceptibility dependent upon differences of weight at this range.)

Intraperitoneally vaccinated guinea pig	weight, ^{gm.} 910
" " " "	" 700
Subcutaneously " " " "	" 800

None of the vaccinated guinea pigs developed typical typhus fever. Two of them—one, perhaps significantly, the subcutaneously vaccinated one—never exceeded a temperature of 103.5°, and were below this for all but 1 day. None of the vaccinated animals developed swelling. The third animal, and—fortunately for conclusions—the heaviest, had an isolated rise of temperature to 105° on the 6th day, and touched 104° on the 8th, but at no time had swelling. This may perhaps indicate a very mild typhus reaction.

The controls all ran typical Mexican fever of a severe type. There was no great difference in the severity of the disease sustained by the lighter control and that occurring in the heavier guinea pigs. In all four controls the temperature was either 105° or almost that on the 4th day, and evidence of swelling was present in all of them on that day, the large as well as the small.

It seems a fair conclusion that the vaccine completely protected two of our animals against the virus given in this experiment and almost completely protected in the third. Also, the very slight difference in the severity of the disease in the light and the heavy controls lends more weight to our previously reported experiments, where this point was the only obvious source of error.

TABLE I
Properties of Partial Hydrolysis Products of S III

Fraction	[α] _D	Barium	Reduction calculated as glucose		Average formula weight	Precipitability by copper ion or rabbit anti-serum	N pptd. by 0.05 mg. from 1 cc. antibody solution*	N pptd. by 0.15 mg. from 1 cc. antibody solution*
			Shaffer-Hartmann method	Willstätter-Schudel method				
	degrees	per cent	per cent	per cent			mg.	mg.
H ₂ SO ₄ B	-27.2	17.2	9.8	10.3	1,800	—	1.21	1.68
H ₂ SO ₄ C	-20.3	17.8	17.1	18.1	1,020	—	1.15	1.30
H ₂ SO ₄ D	-7.2	17.2	32.5		550	—	0.31	
HCl C	-27.0	16.2	12.8	12.5	1,430	—	1.16	1.62
HCl D	-21.9	16.4	15.9	15.0	1,165	—	1.09	1.36
Aldobionic	+10.6	18.8	50.0		360	—	—	—
S III	-34.0				5,600†	++++	1.23	2.12

* Prepared from antipneumococcus horse serum by Felton's procedure (13). Analyses according to (14).

† See Reference 7.

TABLE II
Precipitin Reaction between Hydrolysis Products of S III and Antipneumococcus Horse Serum, Type III

Fractions in order of decreasing molecular weights	Dilution of fraction							
	1:250	1:500	1:1,333	1:2,000	1:4,000	1:10,000	1:40,000	1:1,000,000
S III	(-)	(±)	(+)	(++)	(+++)	(++++)	(++++)	(±)
	±	++	++++	++++	++++	++++±	++++	++
H ₂ SO ₄ B	(-)	(-)	(-)	(±)	(++)	(++)	(+)	(±)
	—	—	+±	++±	+++	++++±	++++	++
HCl C	(-)	(-)	(-)	(±)	(++)	(++)	(+)	(±)
	—	—	+±	++	+++	++++	++++	++
HCl D	(-)	(-)	(-)	(-)	(+)	(++)	(+)	(±)
	—	—	—	+±	++	+++	++++	++±
H ₂ SO ₄ C	(-)	(-)	(-)	(-)	(+)	(+++)	(+)	(-)
	—	—	+	++±	++	+++±	++	++
H ₂ SO ₄ D	(-)	(-)	(-)	(-)	(-)	(++)	(+)	(-)
	—	—	—	±	+±	+++±	++	+
Aldobionic	—	—	—	—	—	—	—	—

Parentheses indicate readings taken immediately after mixing. Other readings after 2 hours at 37°, overnight in ice box.

In another experiment—three vaccinated guinea pigs and three controls—there was practically no protection in two of the vaccinated animals, but the third developed a brief and mild febrile period (3 days) with prompt defervescence, as contrasted with severe and typical courses in the controls. This experiment, too, can be summarized as showing little or no evidence of protection.

In the third experiment—again three vaccinated and three control animals—the results were more definitely indicative of some protective effect on the part of the vaccine. The three controls all developed severe and prolonged typhus infections with temperatures rising to above 104° on the 8th and 9th days and lasting at approximately this level for 11 to 12 days, accompanied by considerable emaciation of the animals. Of the three vaccinated guinea pigs, one seemed completely protected and the other two had brief and mild fever curves distinctly in contrast to those of the controls, lasting for 3 days above 104° . It is worth noting that this experiment was done 107 days after the last administration of vaccine.

DISCUSSION

The experiments recorded above leave no question in our minds that active immunization against the Mexican and endemic American varieties of experimental typhus fever can be carried out with killed and washed vaccines prepared by the X-ray rat method described by us. Whether the method will prove effectual in man remains to be seen, but this problem is being actively investigated by Mexican students of the disease—particularly by Varela, by Casco and by Bustamante. That the vaccine, properly controlled, does no injury has been adequately demonstrated by the fact that these workers have vaccinated many thousand individuals without accident.

We have no data to offer yet as to the lasting qualities of the vaccines, nor do we know whether formalin or phenol (which is used by Weigl for his louse vaccines) is preferable as a preservative. That the protection conveyed by the vaccine is not a very short-lived one is indicated by the fact that in experiments carried out from 36 to 107 days after the last vaccination the results were favorable. If there should still be a lingering doubt in the minds of some investigators as to whether or not the *Rickettsiae* obtained by us from the

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3. Subcutaneous vaccination is fully as effective as intraperitoneal—even when the subsequent infection is intraperitoneal.

4. As in previously reported experiments, the vaccines made with the Mexican organisms conferred only partial and feeble protection against the European virus (Breinl strain).

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STUDIES ON TYPHUS FEVER

X. FURTHER EXPERIMENTS ON ACTIVE IMMUNIZATION AGAINST TYPHUS FEVER WITH KILLED RICKETTSIA*

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(Received for publication, October 26, 1932)

In a number of preceding communications, the writers have published the results of active immunization of guinea pigs with formalin-killed *Rickettsiae* obtained from the tunica lesions of guinea pigs infected with the Mexican virus. Such experiments showed that it was possible to obtain a considerable degree of active immunity against infection with the Mexican virus. Against the European virus this method gave only a partial immunization, amounting often only to a distinct diminution of the severity of the disease.

Since the publication of our earlier results, the methods of vaccine production have been considerably improved by the use of rats in which resistance to typhus had been depressed either by benzol injection or by exposure to radiation with short wave length X-rays. The X-ray method has since been found to be far the more regularly successful one. Intraperitoneal inoculation of animals so treated with moderate doses of Mexican virus yields almost invariably massive amounts of peritoneal *Rickettsiae* (1). This method is now being extensively investigated by Varela and others in Mexico, and its effectiveness in man will, we believe, eventually be determined by these workers. Meanwhile, we have been induced to carry out a new series of immunization experiments on guinea pigs with the use of the X-rayed rat vaccines, since in these preparations there is a much higher concentration of *Rickettsiae* than in any of the others hitherto employed by us.

* This work was aided by a grant from the DeLamar Mobile Research Fund.

Felix reaction at this time showed agglutinations as follows: 1-20, + + + +; 1-40, + + +; 1-80, + + +; 1-160, + +.

A number of protection experiments were done with this serum. Moderate doses of tunica virus were mixed in test-tubes with varying amounts of the immune horse serum ranging from 0.025 to 0.25 cc. Controls with normal horse serum were always carried out. We do not describe these experiments, since the results obtained were not decisive, although in two of them there was indication that the immune serum exerted a distinct inhibitory action upon the virus. In the third experiment of this series, the controls all reacted typically, with temperature and swelling, while of five animals receiving immune serum, one only developed scrotal swelling and this one alone developed a temperature of over 104° at any time.

The increase of the Weil-Felix reaction, an increase which as far as we know has never been noted in animals injected with dead virus alone, and the moderate protective effects encouraged us to continue immunization of the horse since, after all, the amounts of antigen which we had been able to inject were incomparably inferior to those with which one expects to obtain potent antibody production in horses in analogous immunization with bacterial materials.

We therefore, after an interval from the middle of March until the end of May, resumed injections of the vaccine, employing the method advised by Dr. Benjamin White for horse immunization. This consists in giving three injections on consecutive days, followed by intervals lasting about a week. With several interruptions due to the difficulty, at times, of producing enough vaccine, immunization was carried on from May 31 until Aug. 6. The injections were made intravenously. During this period the horse received eighteen injections ranging from amounts of 5 to 10 cc. during the first series, to 25 to 30 cc. of thick suspensions of *Rickettsiae* during the last three series.

On Aug. 15, 9 days after the last injection, a large amount of blood was taken from the horse. At this time the Weil-Felix reaction was + + + + in dilutions of 1-40 and 1-80, + + + in 1-160 and definite, though incomplete, at 1-320. Control normal horse serum never agglutinated the *Proteus* X-19 in dilutions above 1-40. This was true as well of the serum of the immunized horse taken before vaccine injections were begun.

In our preliminary protection experiments, much difficulty was encountered owing to the impossibility of comparative accuracy in the dosages of virus used in consecutive experiments—no two tunica materials containing exactly comparable amounts of *Rickettsiae*. Because of this, and in order to avoid unnecessary expansion of this report, we shall summarize all but the last experiment.

Protection Experiments

1. In two experiments in which amounts of immune serum ranging from 0.05 to 0.2 cc. were mixed with 1/30th to 1/40th of a virulent

The purpose of the present communication is to add to our previous reports the results of active immunization in guinea pigs with this X-rayed rat material killed with 0.2 per cent formalin. We are not at all sure that formalin is better than phenol for preservation, but we have not had time so far to carry out comparative experiments.

EXPERIMENTAL

Preparation of Animals.—Guinea pigs were vaccinated with formalinized rat *Rickettsia* made by the X-ray method containing a relatively small amount of detritus but very large amounts of *Rickettsiae*. The vaccine was not used immediately after being made, but had been kept from 1 to 3 weeks in the ice box.

Fifteen guinea pigs received the vaccine intraperitoneally, and five guinea pigs received it subcutaneously, as follows:

	cc.
May 18.....	0.5
“ 23.....	1.0
“ 28.....	1.0
June 3.....	1.0

No protection experiments were done until July 9, it being desirable for practical purposes to determine whether any acquired immunity would last for at least a minimum of 5 weeks and longer.

Experiment I. July 9, 1932. Inoculation with Mexican Typhus Virus.—The infectious material for this experiment was the tunica scrapings of two guinea pigs, Nos. 1 and 2, killed on the 6th day after intraperitoneal inoculation with tunica and blood from an animal infected with Mexican virus. Three vaccinated animals and two controls received, each, 2 cc. of a suspension of the four tunicas, the dose representing about one-third of a tunica for each guinea pig. Examination of the tunicas after the experimental inoculations had been made revealed that in one of them there were enormous numbers of extracellular *Rickettsiae*, far more than are usually seen in guinea pigs at this stage. These animals, therefore, received tremendous doses. The vaccinated guinea pigs weighed from 80 to 125 gm. more than the two controls, although the largest available control animals were chosen.

The results of this experiment are unsatisfactory, perhaps because of the enormous dosage employed. Nevertheless, there was distinct evidence of a higher resistance in the vaccinated than in the control animals. The controls on the 4th day reached temperatures of 104.2° and 106°, respectively, and typical swelling appeared at that time. One of these control animals, No. 4, was killed on the 6th day, in order to keep the strain going. Its temperature had not gone below 104° since the 4th day and was at that time 104.5°. The other control ran a temperature fluctuating between 105° and 106° from the 4th to the 8th day, with swelling that did not decrease until the 8th and 9th days.

the virus injection. One received normal horse serum 24 hours after infection. One received no serum.

(a) The animals receiving the serum 24 hours after the virus showed a temperature touching 104° for 3 days, swelling for 1 day, but deferescence on the 5th day. The analogous normal serum control had a severe attack of the disease lasting 12 days, with scrotal swelling lasting 5 days.

(b) The animal receiving the serum 48 hours after the virus was completely protected.

(c) The animal receiving the serum 72 hours after the virus was not protected.

The severity of the disease in the controls in this experiment, together with the results of the experiment described in detail below, indicates that our dose of virus was excessive and that, for reasons not clear to us, the subcutaneous method of giving the serum in this type of experiment is preferable to the intraperitoneal route.

In another experiment, two guinea pigs were given a moderate dose of tunica virus intraperitoneally. One of them received 1.5 cc. of the immune horse serum intraperitoneally 24 hours later. The other was similarly injected, at the same time, with normal horse serum. The immune serum animal was completely protected. The normal serum animal sustained a typical and severe attack of typhus.

The following experiment will be described in detail, since we believe that it permits the definite conclusion that the serum of our horse—even at its present relatively low potency—will prevent the development of typhus in guinea pigs, even when administered toward the middle or end of the incubation time.

On Oct. 3, eight guinea pigs of approximately the same weight were given, intraperitoneally, 1 cc. each of washings from a Mexican typhus guinea pig tunica fairly rich in *Rickettsiac* and representing approximately $1/50$ th of a tunica. These animals were paired. One guinea pig of each pair received, subcutaneously, 2 cc. of normal horse serum; the other animal of the pair receiving the same amount of immune horse serum, in the same way. These injections were given to one pair 24 hours after the virus; and to the other pairs 48, 72 and 96 hours, respectively, after the virus had been administered. The reactions of the guinea pigs are set forth in Charts 1 to 4.

It is apparent from this experiment that, with amounts of virus which gave typical and severe typhus fever of the New World variety

We feel safe in assuming that in this experiment, with a moderate dose of infectious material, we obtained practically complete immunity in all three of the vaccinated animals.

*Experiment III. July 18, 1932. Vaccinated Animals Inoculated with Tunica Virus, Eastern United States Variety (Maxcy).—*This experiment was carried out with the Wilmington strain of typhus, that is, the variety discovered by Maxcy to exist in the eastern United States. This disease may be regarded as identical with the Mexican type.

The source of material for infection was tunica material, showing *Rickettsiae*, from a guinea pig inoculated on July 8 with frozen spleen preserved by Dr. Pinkerton at a temperature below zero for about a week. This source animal showed a typical reaction on the 8th day. When killed for removal of the tunica on the 10th day, there was swelling and a temperature of 105° .

Two controls and two treated animals—one vaccinated subcutaneously, the other intraperitoneally—were intraperitoneally inoculated with about one-quarter of a tunica, the material containing relatively few *Rickettsiae*. Apparently the dose was larger than we suspected from the appearance of the tunica, and a tempestuous disease occurred in the two normal controls. These animals developed temperatures of 104° and 105° , respectively, on the 3rd day. This was followed by the customary remission on the 4th day and a return to temperatures above 104° on the 5th day. One of the controls developed typical swelling on the 6th day, the other not until the 8th day, the course of the disease from then on being in every way characteristic. The two vaccinated animals showed no signs of typhus fever. One of them was observed for 2 weeks, during which the temperature rarely went above 103° , never above 103.5° , and there was no swelling at any time. The other touched 104° on the 6th day, but promptly came down and stayed down until the 11th day, a time at which the disease had passed through its entire cycle in the controls. A rise of temperature to 104° on the 12th day in one of these animals was shown to be due to intercurrent pneumonia.

We believe that this experiment indicates complete protection by the vaccine. But again in this experiment we were forced by circumstances to choose controls that were from 100 to 150 gm. lighter than the vaccinated guinea pigs, which had grown heavier during the interval between vaccination and protection tests.

*Experiment IV. July 29, 1932. Inoculation with Mexican Tunica of Animals of Approximately Equal Weight.—*Although past experience has not indicated that, after guinea pigs have reached a weight of 400 or 500 gm., there is any noticeable difference between the large and the smaller ones in susceptibility to typhus infection, nevertheless, we were still a little troubled by the fact that in all the experiments so far recorded we had been forced by circumstances to use controls

in all of the four animals in which normal serum was given, no disease occurred in the three animals in which the immune horse serum had been subcutaneously administered 24, 48 and 72 hours after the intraperitoneal injection.

Even in the animals in which the immune serum was delayed for 96 hours, that is up to one day before the control developed typical fever, the disease seems to have been postponed for 2 days and the swelling limited to a slight reaction lasting for 1 day only.

Effect of the Serum on the European Rickettsia

In these experiments, we have been puzzled and considerably worried by the fact that our immune horse serum, which exerts such unquestionable potency in protecting against the New World disease, has so far exhibited no similar protective action against the European virus. In preceding papers (2) we have reported the analogous observation that active immunization, satisfactorily accomplished against the New World disease with our vaccines, was only partially successful against infection with the European disease. Yet cross-immunizations in animals convalescent from infections with the living virus of these two varieties have been regularly observed by others as well as by ourselves; and cross-agglutinations published by us in a recent paper (3) have indicated a close relationship between the respective agents of the two types of typhus. The question arises whether this may not be a quantitative difficulty which can perhaps be overcome by a more energetic immunization of the horse with living Mexican organisms superimposed upon the treatment with killed *Rickettsiae*.

For obvious reasons, we have been unable to determine the agglutination titer of the horse serum against our own vaccines. The injections made into the horse were carried out with material obtained from the peritoneal exudates of rats, and we have been able to discover no other method of obtaining Mexican *Rickettsiae* in a form suitable for agglutination reactions. *In vitro* serum tests carried out with such an antigen and this horse serum would, therefore, be rendered valueless by the reaction inevitably occurring between rat protein and its antibodies. The Weil-Felix reaction developing in the horse indicates, of course, that a change had taken place in the animal analogous to that which occurs when an attack of typhus fever—either of the Old

The foregoing experiments seem to us to furnish adequate proof that our formalized *Rickettsia* vaccines are capable of producing an active immunity against the Mexican typhus virus as well as against the endemic typhus of Maxcy, provided reasonable doses of the infectious material are used for the protection tests. But even when, as in Experiment I, an overwhelming dose of *Rickettsiae* is administered, there is a prolongation of incubation time, a modification of the disease in the direction of mildness and, occasionally (one animal of three), complete protection.

Although there is an unquestionably close relationship between the infectious agents of the New World typhus and those causing the European disease—as indicated by cross-immunity and overlapping agglutination reactions—there are, nevertheless, considerable differences between the two diseases which prohibit the assumption that a method of vaccination effective against one disease will be equally effective against the other. Recent investigations by Varela and his collaborators (5) appear to indicate that the vaccine prepared by our method is protective against the Tunisian virus. But this virus, as sent to us by Dr. Nigg, has in our hands shown the characteristics of the Mexican-American type,—rapid development of temperature and frequent scrotal swelling. Whether it has become modified since importation, or whether it is intrinsically different from the typical European virus, we are unable to state at present. Meanwhile, it seemed desirable to carry out a few further experiments to determine whether our vaccine possessed any protective properties against the European infectious agent as represented by a strain in our possession—isolated by Breinl and furnished us by the courtesy of Dr. Dyer. This was particularly advisable since in earlier experiments with less potent vaccination materials we found that formalized *Rickettsia* rarely gave complete protection against the European disease, though often it seemed to exert a partially immunizing effect leading to febrile reactions in the treated animals definitely milder and shorter than those observed in controls. One was a Three experiments with European virus were done. One was a complete failure, since the dose of virus used for the tests was inactive and failed to infect the controls.

the development of properties in the horse's serum which may be described as follows:

1. An original agglutinating potency for *Proteus* X-19 (Weil-Felix reaction) not exceeding dilutions of 1-40 was enhanced to a potency of 1-160 and, feebly, 1-320.

2. The horse's serum, after immunization, exerts distinct protective action against the Mexican virus, whether mixed with the virus before injection, administered 1 week or somewhat longer before the virus or given 24, 48 or 72 hours after infection with virus. Experiments of the last category have been most successful when the serum was given subcutaneously.

3. The serum of the immunized horse agglutinates the Weigl louse vaccines, containing *Rickettsia prowazeki* of the European disease in dilutions comparable in potency to the Weil-Felix reaction.

Since the serum described exerted protective effects when given 7 and 13 days before infection with the virus, there is some prospect of prophylactic usefulness on the part of this serum.

Since the serum also protected when subcutaneously administered 24, 48 and 72 hours after intraperitoneal infection of guinea pigs, and somewhat modified the disease even when given 96 hours after infection—that is 1 day before the control came down with a typical reaction—it is at least logical to investigate its possible therapeutic value by early administration in the human disease of the New World or Mexican type. The time intervals governing the experiments of the last two categories in guinea pigs were of course dependent upon the balance between the amounts of virus and of serum used.

The failure of similar effects upon the European virus is not easily explained. This is especially difficult to understand in view of the unquestionable agglutinating potency of the serum for the Weigl vaccines. Whether this difficulty is purely a quantitative one, or depends upon other factors, can be determined only by further investigation.

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tunica vaginalis of guinea pigs represent the true etiological agents of the New World disease, we may point to Experiment II, in which the *Rickettsia* vaccine protected against inoculations with the blood of animals at the height of the disease. To assume in that case that the vaccine did not contain the specific agent would necessitate the corollary that the diseases in the Mexican and North American guinea pigs were not typhus fever at all, but a *Rickettsia* disease quite distinct from European typhus.

Since these experiments, together with our previous ones and those of Kemp and of Dyer and his associates, establish the possibility of active immunization with killed *Rickettsiae*, the ideal method to be aimed at should be the development of cultural methods, either on special media or on tissue, which will yield sufficient quantities of *Rickettsiae* for vaccine production. Until this is achieved, we believe our X-ray rat technique to be the most practically applicable method.

In regard to the European variety of the disease, our results remain partial. In view of the results of Varela and his collaborators, who protected with our vaccines against the Tunisian virus, it may be of value to attempt human vaccination with our materials in Africa as it is being carried out in Mexico. We should hesitate to advise such a procedure in Europe until we have achieved more than the partial experimental successes which we have described. Our results in this regard are definitely inferior to most of those reported in guinea pigs with the Weigl louse vaccine. Nevertheless, they are distinctly encouraging to further effort.

SUMMARY

1. Vaccines consisting of formalinized *Rickettsiae* of Mexican typhus fever, obtained by our X-ray rat method, produce definite resistance in guinea pigs to subsequent infection with the virus of this disease.

2. The resistance so produced amounts to complete immunity when the subsequent infectious dose is moderate—that is, consists of typhus blood or of tunica material in reasonable amounts (not more than one-quarter of a tunica—i.e., roughly 100 to 250 infectious doses). When, as in the first experiment, excessive doses of infectious material were given, the vaccination protection was, in two of the three animals, incomplete.

metabolism. The bile salts may well be under suspicion as integrated in this reaction. It seemed obvious that a careful study of these cycles of pigment overproduction in splenectomized bile fistula animals would furnish valuable information related to the construction of pigments in the body.

Methods

The renal type of bile fistula was used as devised by Kapsinow, Engle and Harvey (3). This type of fistula has been utilized for several years in this laboratory and the care of these dogs with method of analysis of bile pigment has been described in detail elsewhere (5). We emphasize the fact that these dogs with bile flowing freely into the renal pelvis can be maintained in perfect health and weight equilibrium for years. It is necessary to give 50-75 cc. of bile daily, together with a balanced ration.

The spleen may be removed at the time of the bile fistula operation or subsequently. Dogs are kept in metal metabolism cages at all times and water is given by stomach tube about 3 hours before the 24-hour urinary collection is made. The dog usually empties the bladder between the water ingestion and urine collection which makes for uniformity in urine collection as obviously residual urine may give irregular values for daily bile pigment elimination. Catheterization would introduce infection and is never employed. Chloroform 5 cc. placed in the collection bottle acts as a preservative.

The dogs are weighed 3 times each week. Red cell, hematocrit and blood hemoglobin determinations are made at least once a week and often daily during periods of pigment overproduction and anemia (4). Occasional blood plasma volume determinations are done by the vital red method.

Diets are essentially the same in each experiment and consist of canned salmon, "Klim" (a commercial dried whole milk powder) and a bread prepared in this laboratory. Dog 29-353 received a daily diet of salmon bread 300 gm., canned salmon 50 gm., "Klim" 50 gm. and dog bile 50 cc. Dog 30-62 received a daily diet of salmon bread 400 gm., canned salmon 50 gm., "Klim" 30 gm. and ox bile 50 cc. Dog 31-73 received a daily diet of salmon bread 500 gm., canned salmon 100 gm., "Klim" 40 gm. and ox bile 50 cc. Water 400 cc. is added to the dried bread and the ingredients mixed into a mash. The bread is used in our anemia colony and is an adequate diet capable of maintaining dogs in health indefinitely. It contains wheat flour, bran, potato starch, canned salmon, sugar, cod liver oil, canned tomatoes, yeast and a salt mixture. Its preparation has been carefully described (7). On this diet the hemoglobin production of anemic healthy dogs has been carefully studied and is well understood. The obvious advantage of this diet in these bile fistula dogs for bile pigment study needs no comment. An output of 2-4 gm. of hemoglobin each week over and above the maintenance factor is to be expected on this diet.

STUDIES ON TYPHUS FEVER

XI. A REPORT ON THE PROPERTIES OF THE SERUM OF A HORSE IMMUNIZED WITH KILLED FORMALINIZED RICKETTSIA*

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(Received for publication, October 26, 1932)

As a natural corollary of our efforts to produce a vaccine for active immunization against the New World variety of typhus fever, we undertook, over a year ago, to immunize a horse with these vaccines, in order to determine possible antibody production and protective properties.

Course of the Immunization

We began on Sept. 30, 1931, to inject, subcutaneously, into a healthy horse formalinized *Rickettsia* suspensions made from the peritoneal exudates of rats prepared and infected by the benzol technique described by us in preceding papers. These injections were continued until Jan., 1932, for nine injections, the material being only moderately rich in *Rickettsiae*. At the end of that time, the Weil-Felix reaction of the horse, which had originally agglutinated *Proteus* X-19 ++ in 1-40 dilutions, had increased only to ++++ in 1-40 and + in 1-80 dilutions. The injections were intermitted at this time, because the pressure of other work prevented our attending to vaccine production.

In Feb., 1932, we developed a method of reducing the resistance of rats by exposure to X-rays (1), a procedure which we have used for vaccine production since that time and which is being at present extensively used in Mexico in the preparation of material for human vaccination. This method yields a material so rich in *Rickettsiae* that it is possible to inject large amounts of antigen in relatively small volumes, and also to wash the *Rickettsiae*, reducing the content of rat protein.

With this material immunization of the horse was continued until Mar. 18, when a total of nineteen injections of the killed vaccine had been given. On Mar. 24, 8 days after the nineteenth injection, the horse was bled. The Weil-

* This work was aided by a grant from the DeLamar Mobile Research Fund.

TABLE 1

Cycles of Pigment Overproduction

Dog 29-353.

Date	Bile pigment output per 24 hrs.	Hb. level	Urine-bile collected 24 hrs.	Food consumed	Wt.	Remarks
<i>1931</i>	<i>mg.</i>	<i>per cent</i>	<i>cc.</i>	<i>per cent</i>	<i>kg.</i>	
Jan. 22	104	137	520	100	15.6	Splenectomy Sept. 17, 1930
23	74		520	100		
24	74		1100	100		
25	75		930	100		Control period
26	81		900	100	16.2	
27	75		1000	100		
28	76		1300	100		Blood volume 1100 cc.
29	99	136	1100	100	16.0	
Average....	82					
May 7	690	45	480	66	14.1	Maximum pigment output
8	567		710	66		
9	704		580	100		
10	284		470	62		13.3
11	615		625	100		
12	397		570	74		
13	620		665	75		13.3
14	494	44	465	74		
Average....	546					
May 21	199	45	755	100		End of 1st cycle of pigment overproduction
22	196		620	100	12.4	
23	211		540	100		
24	222		760	100		
25	163		640	100		
26	127		610	100	12.7	
27	113		650	100		
28	94	72	640	100	12.7	
29	80		655	100		
30	77		740	100		
31	62		575	100		
June 1	71		660	100	13.4	
2	63		670	100		
3	110		630	100		
4	206	75	735	100	13.5	
Average....	133					

tunica and left at room temperature for 10 minutes before intraperitoneal injection into guinea pigs, we obtained distinct prolongation of the incubation time (4 to 6 days) and shorter temperature curves than were observed in normal horse serum controls. There was a definite effect of the immune serum, but the amounts of serum were obviously too small in relation to the doses of virus.

2. In one experiment in which amounts of 0.1 cc. of the immune serum were mixed with small doses of virus (1/250th of a tunica) and the mixtures allowed to stand for 30 minutes at room temperature before intraperitoneal injection, the immune serum animals were completely protected, while the normal horse serum control sustained a typical typhus fever after a prolonged incubation time of 14 days.

Duration of Passive Immunization

In another experiment, four guinea pigs received 1 cc. each of immune horse serum subcutaneously. Controls were given 1 cc. each of normal horse serum in the same way. Intraperitoneal infection was carried out with a moderate dose of tunica virus 24 hours, 7 days, 13 days and 18 days after serum injection.

(a) When the virus was given 24 hours after the serum, there was complete protection.

(b) When the virus was given 7 days after the serum, the immune serum animal sustained a very mild fever (1 day), with slight atypical swelling, while the control had a severe attack beginning in 3 days and progressing with typical swelling and temperature curve.

(c) When the virus was given 13 days after the serum, the animal which had received the immune serum was partially protected in a manner described in Paragraph (b).

(d) When the virus was given 18 days after the serum, the protective effect of the serum had completely disappeared.

This experiment encourages the hope for possible prophylactic usefulness of sera of this type.

Can the Serum Protect if Given after the Virus?

Five guinea pigs received strong doses of tunica virus intraperitoneally. Three of these animals were given 1.5 cc. each of immune horse serum, intraperitoneally, 24, 48 and 72 hours, respectively, after

TABLE 3

Cycles of Pigment Overproduction

Dog 29-353 (continued).

Date	Bile pigment output per 24 hrs.	Hb. level	Urine-bile collected 24 hrs.	Food consumed	Wt.	Remarks
1931	mg.	per cent	cc.	per cent	kg.	
July 22	429	43	625	100	14.6	Maximum bile pig- ment output
23	385		835	100		
24	485		640	100	15.1	
25	422		560	100		
26	606		730	100		
27	462		550	100	14.4	
28	760		730	100		
29	878		630	100	14.7	
30	798		835	100		
Average....	581					
Sept. 24	130	71	745	100	14.5	Low pigment out- put
25	102		850	100		
26	85		695	100		
27	90		615	100		
28	101		785	100	14.5	
29	82		515	100		
30	88		830	100		
Oct. 1	94	95	825	100	14.5	
Average....	96.5					
Oct. 7	181	99	710	100	15.0	Blood volume 1057 cc.
8	266		760	100		
9	221		700	100	15.3	
10	230		960	100		
11	202		740	100		
12	220		1150	100	15.0	
13	193		445	100		
14	230		850	100	15.0	
15	127		790	100		
Average....	208					

Death Oct. 24, 1931. Autopsy given below.

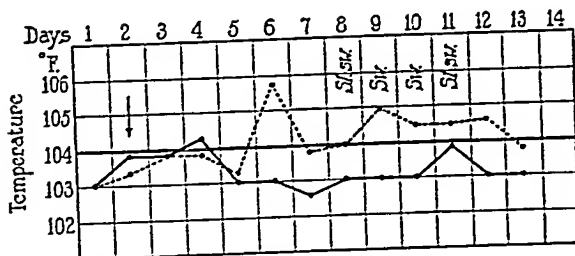


CHART 1

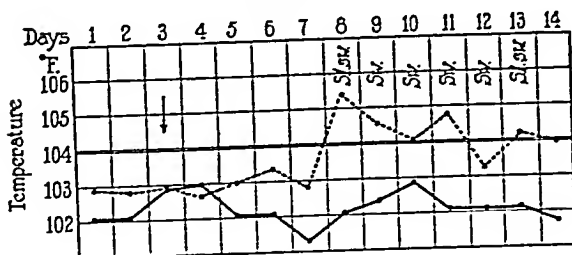


CHART 2

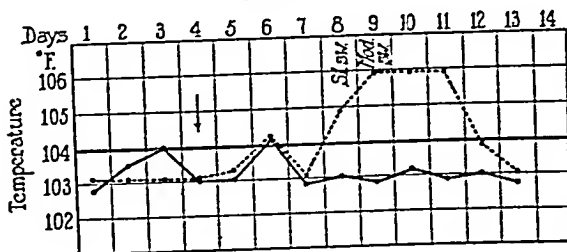


CHART 3

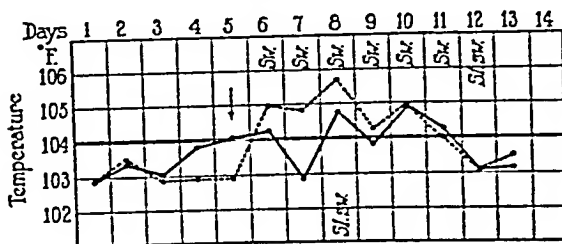


CHART 4

CHARTS 1 to 4. In these charts the heavy black lines represent the temperature reactions of the animals receiving the immune serum; the broken lines those of the animals receiving equivalent amounts of normal horse serum. The arrows indicate the points at which the serum, normal or immune, was injected. The letters "sw." at the tops of the charts indicate typical scrotal swelling in the normal horse serum controls; at the bottom of Chart 4, in the animal treated with immune serum. All animals received virus intraperitoneally on the 1st day.

consideration of the total bile pigment overproduction. This total production of bile pigment above the control level is also expressed in column 4 as grams of hemoglobin obtained by calculating 40 mg. of bile pigment as equivalent to 1 gm. hemoglobin.

Pigment lost to the body is expressed in columns 7 and 8 in terms of hemoglobin as coming from the urine bile pigment above the control levels—columns 4 and 7 are equivalent. The pigment lost from the blood (column 8) is estimated from the fall in blood hemoglobin levels during any given period—the blood volume \times 13.8 \times per cent hemoglobin lost. Hemoglobin 100 per cent = 13.8 gm. per 100 cc. whole blood.

Pigment gained for the body is given as grams of hemoglobin in columns 9 and 10. Column 10 records the gain from transfusions. Column 9 records the gains in the hemoglobin level in the blood during any given period and the grams hemoglobin estimated exactly as were the grams hemoglobin lost. This new formed hemoglobin is presumably derived at least in part from the diet intake.

Unexplained "X" pigment surplus is given in the last column expressed as hemoglobin grams equivalent whether coming from hemoglobin or bile pigment. It is the sum of the surplus bile pigment expressed in hemoglobin equivalents (column 4 or 7) plus any hemoglobin gained in the blood by rise of hemoglobin per cent (column 9) less any hemoglobin given by transfusion (column 10) and less any hemoglobin lost from the blood stream (column 8) as presumably this would go direct to form bile pigment and therefore is "explained."

The total *unexplained pigment surplus* amounts to 1113 gm. hemoglobin equivalent whether appearing as hemoglobin or bile pigment. This amounts to 5.8 gm. hemoglobin equivalent per day or 40.6 gm. per week.

A standardized anemic dog on this ration will put out 2-4 gm. hemoglobin per week over and above the unknown maintenance factor. This output of 40.6 gm. hemoglobin equivalent per week approximates closely the high levels of hemoglobin production in anemic dogs when fed liver or kidney diets.

The *second dog* (30-62) was a young female mongrel setter about 1 year of age. Biliary occlusion was incomplete which necessitated a second operation. She began a cycle of pigment overproduction 5 weeks after the second operation and 4 months following the splenectomy. She lived 89 days after the pigment overproduction began. The dog went through 4 cycles of pigment overproduction of 13 to 27 days duration with short periods of low bile pigment output between cycles. She showed icterus during the last 2 months of life. Appetite, food consumption and weight were not disturbed. Two transfusions were given during periods of dangerously low blood hemoglobin levels.

World or the New World variety—has been successfully overcome. Meanwhile, however, we had another reaction at our disposal to throw light on the question discussed—namely, the possible agglutinative properties of this typhus-immune horse serum against the Weigl vaccines, which consist in the triturated intestinal walls of typhus-infected lice containing large quantities of *Rickettsia prowazeki*. We accordingly set up an agglutination reaction with these materials, as follows:

Weigl Vaccine Agglutination
(October 18, 1932)

Dilution	Serum of horse treated with Mexican <i>Rickettsiae</i>	Normal horse serum
1-10	++++	
1-20	++++	—
1-40	++++	—
1-80	++++	—
1-160	++++	—
1-320	++++	
1-640	++++	
	++	

Reactions well started in an hour at 40°C.; complete in 3 hours.

This experiment demonstrates that the serum of our horse, which had received vaccines containing killed Mexican *Rickettsiae*, had developed potent agglutinins against the *Rickettsiae* that occur in lice infected with blood from the European disease.

Such results reinforce the conclusions drawn by us from our previous agglutination studies to the effect that there is a close antigenic relationship between the agents giving rise to the New World and the Old World diseases, respectively. They also encourage the hope that if we can considerably enhance the protective power of our horse serum by further immunizations with living virus, we may eventually obtain a certain amount of protective power against moderate doses of the European virus.

DISCUSSION AND CONCLUSIONS

The experiments recorded above demonstrate that the systematic treatment of a horse with formalin- and phenol-killed *Rickettsiae* obtained from the Mexican virus by our X-ray rat technique induces

Table 5 shows the control level of bile pigment output as 75 mg. per 24 hours. The control hemoglobin level is lower than normal and probably related to the second operation. Following this operation there was a small stitch abscess and some drainage from the wound.

The periods of high pigment output average 414 and 409 mg. per 24 hours—about six times the control level. The blood hemoglobin levels show considerable decrease.

Table 6 is a summary of the pigment output of the second dog (30-62). This type of table is explained under Table 4. The base

TABLE 6

Unexplained Pigment Surplus in the Splenectomized Bile Fistula Dog 30-62. Basal bile pigment output 72 mg. per day.

Days of experiment	Total bile pigment output			Blood Hb. level		Pigment lost gm. Hb. equivalent		Pigment gained gm. Hb. equivalent		"X" pigment surplus
	Total	above control	expressed as Hb.	start	end	in urine	from blood	in blood	transfusion	
	mg.	mg.	gm.	per cent	per cent					gm.
16	2810	1658	41	107	86	41	31			10
21	7992	6480	162	86	27	162	88		25	49
18	6183	4887	122	27	43	122		24		146
19	5834	4466	112	43	38	112	8			104
9	1187	539	13	38	81	13		64	34	43
6	1147	715	18	81	72	18	13			5
89	25153	18745	468			468	140	88	59	357
Per day	283	211	5.3			5.3	1.6	1	0.66	4

bile pigment output is the average of all control observations—72 mg. per 24 hours. The surplus of bile pigment above the control level is expressed as hemoglobin equivalent in columns 4 and 7. This amounts to 211 mg. bile pigment per day or 5.3 mg. hemoglobin equivalent. When we allow for the hemoglobin destroyed to account for the fluctuations in the blood hemoglobin levels we find the "unexplained X" pigment surplus as 357 gm. equivalent of hemoglobin or 4 gm. per day.

When we recall that the standard anemic dog on this diet can produce only 2-4 gm. of hemoglobin a week, this figure of 28 gm. of hemoglobin equivalent becomes a conspicuous figure, not as high as the

SPLENECTOMY IN BILE FISTULA DOGS BILE PIGMENT OVERPRODUCTION, ANEMIA AND INTOXICATION

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The essential features of the experiments tabulated below may be described in a few words. A *splenectomized* dog can be made anemic by bleeding and continued in this condition in our anemia colony for years and will remain in perfect health. Moreover the output of *hemoglobin* on various diets will be identical with that of a dog which retains his spleen and there is no icterus. A dog with a *bile fistula* opening into the renal pelvis will remain in perfect health, in weight equilibrium and without anemia or intoxication provided the diet intake is suitable. Such dogs in this laboratory have been under observation and live in health for several years.

When we *combine splenectomy with this bile fistula* we observe an unexpected physiological reaction. After a time (weeks or months) the dog begins to put out large surplus amounts of bile pigment and the hemoglobin falls but not in adequate measure to explain this large surplus of bile pigments which in some periods may reach 8-10 times control values. This large bile pigment excess output may appear in cycles of days or weeks with intermissions. Anemia may become severe and call for transfusions. Finally a tendency to hemorrhage may develop and cause death due to bleeding into the serous cavities, lungs or gastro-intestinal tract. There is inevitably a lethal outcome in weeks or months when one combines a bile fistula with splenectomy. This is an intriguing riddle and its solution promises a better understanding of the complex internal metabolism of body pigments. That the spleen is essential to life in a bile fistula dog is not without interest and suggests some problematical contribution of the spleen to internal

figure of Table 4 but far beyond any possibility of error in observation or technique.

The *third dog* (31-73) was an adult male mongrel bull dog. The clinical story of this dog is the shortest of the three. He went into an acute cycle of bile pigment overproduction less than 7 weeks after operation and lived only 79 days subsequently. The periods of anemia developed very acutely and called for transfusions. Icterus was not noted until a few days before death. Food consumption was good and there was some gain in weight. In all 11 transfusions were given and the total grams of hemoglobin so introduced were 286 (Table 8).

TABLE 8

Unexplained Pigment Surplus in the Splenectomized Bile Fistula

Dog 31-73. Basal bile pigment output 154 mg. per day.

Days of experiment	Total bile pigment output			Blood Hb. level		Pigment lost gm. Hb. equivalent		Pigment gained gm. Hb. equivalent		"x" pigment surplus
	Total	above control	expressed as Hb.	start	end	in urine	from blood	in blood	transfusion	
	mg.	mg.	gm.	per cent	per cent					
20	11063	7983	200	51	66	200		29	108	121
14	1700	-456	-11	66	101	-11		69		58
17	8110	5492	137	101	32	137	135			2
9	4315	2929	73	32	73	73		80	65	88
19	16422	13496	337	73	36	337	72		113	152
79	41610	29444	736			736	207	178	286	421
Per day	527	373	9.5			9.5	2.6	2.3	3.6	5.5

Table 7 shows a control period which is not adequate as probably the rise in bile pigments due to splenectomy had already started. The appearance of the pigment overproduction was unusually early and acute. We give as a base line bile pigment production 154 mg. per day but have a conviction that the true control level was nearer 100 mg. Even with this high base line the excess of bile pigment output is extreme and on two occasions exceeds 1 gm. per day. There was a sharp drop in the blood hemoglobin which called for transfusions but the dog remained in excellent condition.

Table 8 gives the usual summary for Dog 31-73. This type of table is explained under Table 4. The daily excess of bile pigment output above control is recorded as 373 mg. or the hemoglobin equivalent as

The analysis of bile pigment in the urine is described elsewhere (5) and is done in duplicate.

EXPERIMENTAL OBSERVATIONS

Some years ago Hooper and Whipple (2) observed cycles of bile pigment overproduction in splenectomized bile fistula dogs. The *open bile fistula* was used in those experiments and the possibility of infection as responsible for a part of the reaction could not be excluded. In the experiments tabulated below we believe that infection is excluded as a causative or contributing factor. The experiments of Hooper and Whipple were also less complete as the daily bile collection period was but 6 hours while the present experiments cover all bile pigment eliminated from the body during 24 hours. The hemoglobin levels in the blood are also followed carefully with necessary blood volume measurements. The pigment forming capacity of the diet is also well understood and we can approximate a knowledge of the maximum hemoglobin pigment output of the anemic dog on this diet.

A considerable number of splenectomized bile fistula animals have been observed. We report in detail the pigment studies on 3 of these dogs in which the data on pigments, diet, hemoglobin level and anatomical conditions are reasonably complete. The general pigment reaction is uniform and the anatomical findings very similar which gives one confidence that this is not a physiological curiosity of rare occurrence but a uniform reaction under these fixed conditions.

The *first dog* (29-353) presents a number of cycles of pigment overproduction—in all a continuous period of observation of 190 days. This dog was an adult male mongrel collie. For 7 months following the operation (bile fistula and splenectomy) this dog was used for dietary experiments and hemoglobin injection. A hematoma developed at the site of an injection with softening so that pigment observations were discontinued for several weeks. The precise date at which the bile pigments showed a rise is not known. Regular observations were begun 7 months after the splenectomy and continued until death 190 days later. During this period of observation 6 cycles of pigment overproduction were observed of which 3 are given in Tables 1-3. The shortest cycle was 14 days and the longest 31 days in duration. After the first cycle the hemoglobin never rose to the control levels nor did the pigment output ever fall to normal for more than a few days at a time.

Table 1 shows daily observations of bile pigment output during a port control period—average 82 mg. bile pigment per 24 hours.

Third dog (31-73)—Tables 7 and 8.

This dog ran a very acute course with severe anemia requiring a considerable number of transfusions. Death obviously was due to anemia associated with blood destruction of unknown origin. There was no evidence of bleeding internally or externally.

Autopsy was done immediately after death. Well formed clots were present in heart chambers. All the serous cavities were smooth and glistening. No hemorrhages. Tissues show only a slight icteric tinge.

Heart: Normal in gross. *Histological sections* show a few cellular scars. Heart muscle cells are normal.

Lungs: Normal in gross and histologically.

Gastro-intestinal tract: Normal throughout in gross as well as histologically. No phagocytes containing yellow pigment observed in any sections of the intestinal tract.

Liver: Is dark brown in color, shows a regular architecture. *Histologically* the central part of the lobules shows atrophy of liver cells and here we notice many large phagocytes which contain granular yellow pigment. Some of this pigment gives a positive reaction for iron. The liver cells in the outer half of the lobule are relatively normal. Between these two zones the liver cells in the central zone show conspicuous bile canaliculi filled with dark brown hyalin material. Kupffer cells are numerous and large in all parts of the lobule. Bile ducts are all clean and normal looking. There is no evidence of any infection. Portal tissues show a few mononuclears, some of which contain pigment obviously related to the phagocytes present in the liver lobule and in the hepatic lymph glands and represent a transport of material from the liver through the lymphatics to the hepatic lymph glands. No polymorphs are seen anywhere. No fat droplets can be stained within the liver lobule.

Kidneys: The left kidney is somewhat hypertrophied and pigmented. The right is scarred at the site of the operative incision and about half normal size.

Histological sections: The *gall bladder* epithelium within the renal pelvis is quite normal and retains its familiar characteristics. No evidence of infection. Some phagocytes contain old blood pigment. The kidney parenchyma is normal histologically. The convoluted tubules within their epithelium show coarse yellow pigment as in the other cases. It gives no stain for iron nor for fat. An occasional cast is seen in a collecting tubule. Glomeruli normal. Urinary bladder normal.

Lymph nodes: The hepatic lymph nodes are enlarged and deep brown in color, rather soft and moist. The retroperitoneal nodes are moderately enlarged and light brown in color. Other lymph nodes are normal. The pigmented lymph glands histologically show many phagocytes in the sinuses which contain pigment granules, some of which stain for iron. No marrow cells are observed but some phagocytes contain red cells.

Bone marrow of the femora and humeri is dark red throughout. The tibiae contain a good deal of fat along with the red marrow. The bony substance is

During a cycle of bile pigment overproduction, this average value rises to 546 mg. per 24 hours or about seven times normal. The blood hemoglobin has fallen meanwhile and there was some icteric staining of the tissues. The last part of Table 1 shows the end of this particular

TABLE 2

Short Cycle of Pigment Overproduction
Dog 29-353 (continued).

Date	Bile pigment output per 24 hrs.	Hb. level	Urine-bile collected 24 hrs.	Food consumed	Wt.	Remarks
1931	mg.	per cent	cc.	per cent	kg.	
June 18	86	86	500	100	14.6	
19	90		780	100		
20	76		600	100		
21	66		435	100		
22	91		900	100	14.3	
23	110	93	875	100		
24	150		685	100		
25	257		660	100		
26	422		840	100	14.0	
27	484		690	100		
28	662		660	100		
29	631		700	100		
30	523	28	585	100	13.9	
July 1	405		860	100		
2	403		790	100		
3	294		585	100		
4	165		640	100		
5	219		630	100		
6	138		1235	100		
7	110	66	100			
Average...	269		100	14.6		Blood volume 1100 cc.

cycle of pigment overproduction. The fall to normal is of but short duration and the bile pigment values begin to rise again. There is some loss of appetite and weight during this cycle and this is noted in almost all dogs. Spontaneous recovery from the anemic level took place in all the cycles recorded. This dog did receive one transfusion at a time when his hemoglobin level was dangerously low. Table 2 shows a short cycle of bile pigment overproduction with a

the final analysis as both bile pigment and the pyrrol aggregate contain four pyrrol rings. Possibly both reactions may take place under these conditions.

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short fore period of normal output. There is a sharp drop in blood hemoglobin but spontaneous recovery took place and food consumption and the weight curve remained constant. Icterus was observed during this cycle.

Table 3 shows some very high values for bile pigment for 24 hours—an average of 581 and one day of 878 mg. bile pigment—more than ten times the control level. During this period there is actually a gain

TABLE 4
Unexplained Pigment Surplus in the Splenectomized Bile Fistula
Dog 29-353. Basal bile pigment output 78 mg. per day.

Basal bile pigment output 78 mg. per day.											
Days of experiment	Total bile pigment output			Blood Hb. level		Pigment lost gm. Hb. equivalent		Pigment gained gm. Hb. equivalent		"X" pigment surplus	
	Total	above control	expressed as Hb.	start	end	in urine	from blood	in blood	trans-fusion		
mg.	mg.	gm.	per cent	per cent					gm.		
45	19512	16002	400	60	72	400		18		382	
5	353	-37	-1	72	75	-1		4	36	3	
15	4474	3304	83	75	86	83		17		100	
5	409	19	0.5	86	93	0.5		11		12	
15	4973	3803	95	93	66	95	41	20		54	
51	20915	16937	423	66	79	423		17		443	
13	2041	1027	26	79	90	26				43	
13	2587	1573	39	90	71	39	29			10	
9	822	120	3	71	95	3				39	
19	2906	1424	36	95	89	36				27	
190	58992	44172	1104.5		1104.5	79		36		1113	
Per day	311	233	5.8		5.8	0.42	0.65	0.19		5.8	

in blood hemoglobin—truly a remarkable pigment output. Food consumption and the weight curve remained normal. There was some icterus. The middle period of low pigment output does not fall to the control level.

Table 4 is a condensed table giving a summary of pigment data throughout the entire period of observation. The various periods run from 5 days to 51 days and represent arbitrary divisions in which conditions were relatively similar. Some periods show high or low or intermediate pigment output. The high periods are obvious from a

white were injected intravenously at hourly intervals in four dogs. Before each injection a sample of blood was obtained and a precipitin reaction carried out with immune rabbit serum. Fig. 2 illustrates the results obtained and shows that there is little tendency for the accumulation of egg white in the circulating blood. The serum dilutions positive, 1/16, obtained in Dogs 3 and 4, 5 minutes after the injection of

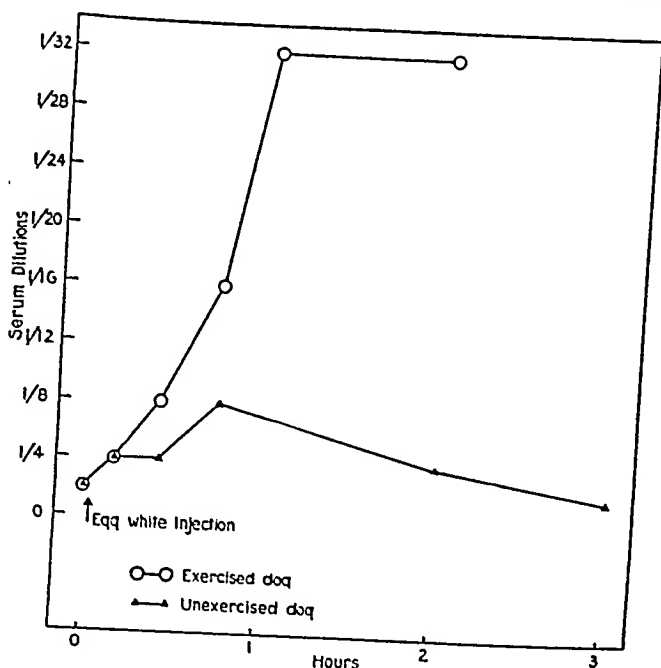


FIG. 1. Experiment I. The concentration of egg white in the blood stream following injection into the knee joint of 3 cc., showing the relative absorption from passively exercised and unexercised joints.

the egg white, agree quite closely with the theoretical concentration in the blood stream at that particular moment, as obtained from calculations. The rapid fall in the next 55 minutes is probably largely due to excretion through the kidney. This explanation is in accord with the finding of Opie (9) that egg white injected subcutaneously soon appears in the blood stream and shortly thereafter can be demonstrated in the urine. This rapid removal of foreign protein from the blood stream may well explain the inconsistent results obtained in later experiments, in some of which we observed no significant appearance of egg white in the blood stream of either exercised or non-

TABLE 5
Cycles of Pigment Overproduction

Dog 30-62.

Date	Bile pigment output per 24 hrs.	Hb. level	Urine-bile collected 24 hrs.	Food consumed	Wt.	Remarks
1931	mg.	per cent	cc.	per cent	kg.	
Aug. 13	72		715	100	10.6	Control period
14	76		745	100		
15	84		725	100		
16	107		1050	100		
17	66		960	100		
18	68		670	100	10.6	
19	70		640	100		
20	56	103	425	100	10.7	
Average....	75					
Sept. 24	430					Blood volume 1081 cc.
25	363	41	630	100		
26	362		485	100		
27	365		670	100	11.1	
28	352		1180	100		
29	341		500	100		
30	405		575	100		
Oct. 1	529	35	665	100	10.8	High pigment output
2	550		625	100		
3	414		615	100		
4	444		615	100		
Average....	414	25	800	100	11.1	
Oct. 22	412					
23	425	43	640	100		
24	430		720	100		
25	438		920	100	11.5	
26	391		955	100		
27	357		1000	100		
28	434		750	100	11.5	High pigment output
29	379		800	100		
Average....	409	58	810	100	11.6	

Death Nov. 28, 1931. Autopsy given below.

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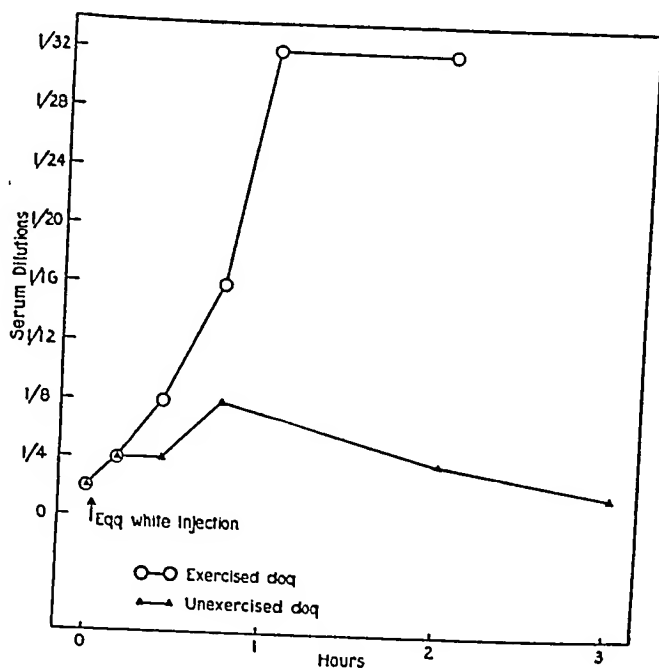


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TABLE 7
Cycles of Pigment Overproduction

Dog 31-73.

Date	Bile pigment output per 24 hrs.	Hb. level	Urine-bile collected 24 hrs.	Food consumed	Wt.	Remarks
<i>1931</i>	<i>mg.</i>	<i>per cent</i>	<i>cc.</i>	<i>per cent</i>	<i>kg.</i>	
Nov. 25		119		100	15.1	Splenectomy Nov. 5, 1931 Control observation
26				100		
27	111		920	100		
28	153		870	100		
29	165		780	100	15.8	
30	173		970	100		
Dec. 1	77		850	100	15.7	
2	228		1280	100		
3				100	15.5	
Average....	151	116				
Dec. 31						
<i>1932</i>						
Jan. 1		51		100	17.2	Transfused 4 times. Total 103 gm. hemoglobin
2	785					
3	526		980	100		
4	950	29	1100	84		
5	950	37	900	95		
6	1096	27	700	90	17.5	
7	1147	40	920	48		
8	782	54	820	100		
	580	55	910	100	17.1	
			810	100		
Average....	852				17.6	Blood volume 1410 cc.
Feb. 7						High pigment output
8	523	89				
9	441	78	770			
10	290	80	960	100		
11	225	78	1020	100	18.6	
12	211	77	960	100		
13	233	65	840	100	18.5	
14	189	69	980	100		
15	349	73	840	100	18.9	
16	449	60	750	100		
17	400		880	100		
18	615	48	800	100	18.7	
19	837	52	860	100		
20	795	46	780	100	19.2	
	849	44	900	100		
Average....	458		890	100	19.3	

Autopsy given below.

one of the blood samples from either dog gave a positive precipitin reaction after the intraarticular injection. It is further seen that the egg white appears in the thoracic duct lymph of the exercised dog in a significant dilution (1/16) in 30 minutes and thereafter rises rapidly, being detected in a dilution of 1/512 at the end of 2 hours. No trace of egg white was detected in the lymph collected from the unexercised dog

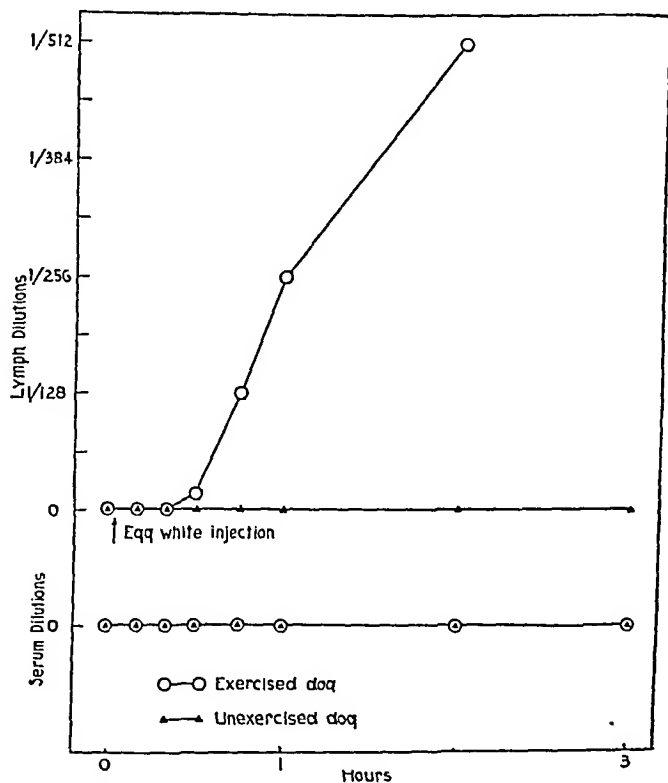


FIG. 3. Experiment IV. The concentration of egg white in the blood stream and thoracic duct lymph following injection into the knee joint of 3 cc. In this case, the leg of the unexercised dog was not massaged.

dog. From these experiments we conclude that proteins such as are contained in egg white are removed from the joint only by way of the lymphatics and if all connections between the lymphatic and vascular systems are eliminated, none of the injected protein will appear in the blood stream. We also believe that this type of experiment demonstrates very clearly the importance of exercise in the removal of proteins from a normal joint.

9.5 gm. When we allow for the transfused blood and the loss of circulating blood hemoglobin, we find 5.5 gm. hemoglobin equivalent per day as the "unexplained" pigment surplus. This amounts to 38.5 gm. hemoglobin equivalent per week and we recall that anemic dogs on this diet can regenerate only 2-4 gm. hemoglobin per week above the maintenance factor. This is a very large excess of bile pigment or hemoglobin to explain.

AUTOPSY PROTOCOLS

First dog (29-353)—Tables 1-4 above.

The final period of intoxication was associated with bleeding from puncture wounds of veins. Whole blood given intramuscularly failed to check it. Bloody stools appeared and transfusions had little effect.

Autopsy done immediately after death. All tissues showed definite icteric staining. There was no fluid or blood in the serous cavities.

Heart: Shows extensive hemorrhage under endocardium of left ventricle. *Histological sections* normal. The hemorrhagic areas in vein walls and fat show no white cells and no evidence of infection.

Lungs: Are crepitant and show many small hemorrhagic spots. *Histological sections:* Many alveoli contain red blood cells, a little fibrin and some polymorphs. There is definite edema. Bronchi are clear. Many alveoli are normal. There is evidently a slight amount of inflammation in some areas.

Gastro-intestinal tract: Stomach normal. Jejunum and ileum show red injected tips of villi and there is partly digested blood in the lumen. There are numerous pinpoint hemorrhages in the colon. *Histological sections:* Stomach normal. Jejunum shows normal mucosa. No evidence of inflammation. There are fresh red cells in the crypts. At the base of the mucosa are many mononuclear phagocytes which contain a yellow granular pigment. These same phagocytes are found also in the tips of the villi. They are a conspicuous feature of the section. Iron stains show no reaction in the majority of these phagocytes but in the tips of some of the villi some of this pigment gives a positive stain for iron. Colon normal.

Liver: In gross the color is a dark reddish brown. The lobules are rather indistinct. There is no evidence of scar tissue. *Histologically* the outer half of the lobules presents normal liver cells. The central part of each lobule shows much atrophy of liver cells and an accumulation of large phagocytes packed with granular yellow pigment, some of which gives a positive stain for iron. The mid-zone of each lobule shows conspicuous bile canaliculi within the liver cells filled with dark brown colloid material. Kupfer cells are numerous in all parts of the lobule and enlarged. Fat stains show no fat droplets anywhere. Bile ducts are clear and normal. No evidence of infection. In the portal stroma are mononuclears and pigmented phagocytes of the same nature as those noted in the center of the lobule.

time in the thoracic duct lymph and that the actual removal from the joint must take place much sooner. Thus, in the preceding experiments, where the lymphatics were not tied off, any appearance of egg white in the blood stream must represent a prior passage through the lymphatics to their point of entrance into the great vessels of the neck. Furthermore, the apparent stagnation of the egg white in the lymphatics of the non-massaged, unexercised leg affords further explanation of the inconsistencies in the first group of experiments dealing with the appearance of egg white in the blood stream alone. In such experiments, no attempt was made to massage either the abdomen or leg. Therefore, we can further conclude that the amount of protein in the thoracic duct lymph in a dog with an unexercised injected knee joint is greatly increased if the leg muscles are massaged.

Experiments in Which Horse Serum Was the Protein Used

Preparation of the Immune Sera.—In the preceding experiments, the egg white used represented of course a mixture of several proteins, with no clue presented as to any possible qualitative or quantitative difference in ease of removal among them. It was determined, therefore, in the case of horse serum, to study comparatively the egress of certain of the protein fractions, by preparing rabbit sera immune not only against horse serum as a whole, but also against its constituents.

Three protein fractions were prepared according to the method of Doerr and Berger (10), with saturated ammonium sulfate from 500 cc. of horse serum.

1. 0-33 per cent saturation $(\text{NH}_4)_2\text{SO}_4$ = euglobulin
2. 33-50 " " " " = pseudoglobulin
3. 50-56 " " " " = discarded
4. 56-66 " " " " = albumin

The euglobulin was precipitated in $\frac{1}{2}$ saturation of the whole serum with ammonium sulfate. The filtrate from that was $\frac{1}{2}$ saturated with ammonium sulfate to precipitate pseudoglobulin and so on. Each precipitate was washed with the corresponding concentration of ammonium sulfate, until the filtrate gave no test for protein. The wash water was thrown away each time. The fraction between 50 to 56 per cent was also thrown away, in order to separate globulin and albumin more carefully. Each precipitate was dissolved in 250 cc. distilled water and reprecipitated by the addition of the proper amount of saturated ammonium sulfate. They were again washed and the washings discarded. Then they were dissolved and dialyzed—euglobulin against 4 per cent sodium chloride changed twice a day and the others against tap water. When

hard and strong. *Histological sections* of the humerus show almost complete cellular hyperplasia replacing all fat. We see a moderate number of phagocytes containing a coarse brown pigment which gives a positive stain for iron. All marrow cells appear normal. Megakaryocytes appear in normal numbers. Femur sections show a good deal of fat remaining, making up about half the section. Tibiae show more than half of the section made up of fat. Wherever the marrow cells occur they are normal. Phagocytes are scattered about uniformly in all sections including those of the vertebrae. This is a moderate grade of hyperplasia, not as extreme as the preceding case.

DISCUSSION

Death in these splenectomized bile fistula animals is due to anemia, usually associated with bleeding into the tissues, intestines or from vena punctures. The blood clots slowly whether in the test tube or vascular system or combined with normal blood. This suggests the presence of an anticoagulant in this abnormal blood. We have not been able to demonstrate that these tissues contain any substance which inhibits the clotting of normal blood plasma.

Blood destruction is an important factor in this abnormal condition and obviously is related to some interrelation of a spleen factor and a bile factor. The bile salts certainly deserve careful scrutiny as we are accumulating evidence that certain of the intoxications related to bile fistulas can be prevented by a proper intake of bile salt by mouth. The spleen factor can be tested by the use of various spleen fractions given by mouth or by vein. We hope to report on this point in the near future.

We expect to study factors which may be related to the blood destruction. It may be suggested that the red cells are produced with inherent defects because of faulty stroma. The marrow looks hyperplastic and is normal as to cell detail. The plasma will be studied to reveal the presence of any factor capable of laking normal red cells. Because transfused normal red cells do not persist in the circulation of these dogs, some hypothetical hemolysin may be suspected.

Muscle hemoglobin does not seem to enter into the picture. There is no conspicuous loss of muscle hemoglobin during the course of this pigment overproduction in these dogs. The striated muscle is normal in gross and histologically at autopsy.

Can we explain all this excess bile pigment overproduction on the

joint, as shown by its high concentration in lymph (positive in a dilution of $1/2,048$), $\frac{1}{2}$ hour after the intraarticular injection in the exercised dog and after 2 hours in the unexercised dog. In contrast, the pseudoglobulin fraction makes only a very slight appearance in the

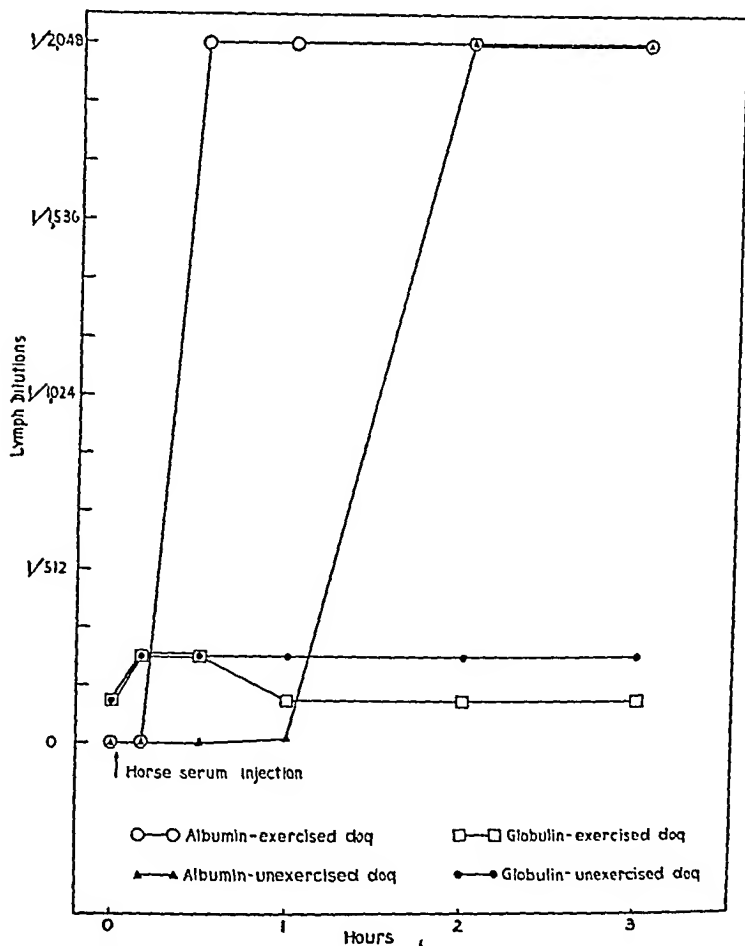


FIG. 5. Experiment VI. The concentrations of albumin and globulin fractions of horse serum in the thoracic duct lymph after injection into the knee joint of 3 cc. undiluted horse serum. Concentrations in the serum showed no significant rise and are omitted from the chart.

thoracic duct lymph of both dogs. This appearance, furthermore, may be only apparent, because the serum used, while acting preponderantly against pseudoglobulin, showed also a noticeable reaction with albumin, due to incomplete separation of the two fractions. Be-

THE MANNER OF REMOVAL OF PROTEINS FROM NORMAL JOINTS*

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Our knowledge concerning the physiology of normal joints is very meager. Fisher (1) concluded from certain of his experiments that true solutions are absorbed from normal joints by the lymphatics and capillaries alike. Key (2) and Rynearson (3) state that particulate matter (India ink) is removed from normal joints by the lymphatics with the aid of fixed and wandering phagocytic cells. Except for a few publications of this sort, review of the literature reveals little concerning the physiology of normal articular cavities. Yet it is obvious that if we had a clearer conception of the factors involved in the interchange of true and colloidal solutions in normal joints, our knowledge of the physiology of these structures would be greatly enhanced and we would better understand the production and maintenance of joint effusions.

The protein which is present in normal and pathological synovial fluids is obviously an important factor in the interchange of normal synovial fluid and the maintenance of articular effusions by reason of its osmotic pressure. We have been unable to find any data in the literature bearing directly upon the removal of colloidal matter like protein from the synovial cavity, although the experiments of others (Lewis (4), Bolton (5), Field and Drinker (6)) have established that proteins such as those contained in horse serum are absorbed from the tissue spaces and serous cavities under normal conditions by lymphatics alone, a belief expressed by Starling (7) in 1896. The purpose of this communication, then, is to set forth experiments under-

* This is Publication No. 10 of the Robert W. Lovett Memorial for the study of crippling disease, Harvard Medical School, Boston.

difficulty, if at all. The experiment also demonstrates the effect of exercise on the rapidity of removal of another protein, horse serum albumin. Finally, as shown by negative tests with the serum samples, horse serum albumin resembles the proteins in egg white in being removed from joints by way of the lymphatics alone. (See protocol, not on chart.)

Experiment VI. (Fig. 5).—

Dog 13, weight 21.8 kilos.

9:20 a.m. Anesthetized with 0.87 gm. nembutal intraperitoneally.

10:20 a.m. Control blood sample removed from right leg vein. Lymphatic trunks were then identified and tied off, and a cannula inserted into the thoracic duct.

1:20 p.m. Control lymph sample secured.

1:22 p.m. 3 cc. horse serum injected into left knee joint and leg exercised passively throughout experiment at rate of 40 to 60 revolutions per minute, and abdomen massaged.

Collections of blood and lymph were made as before at 1:32, 1:53, 2:22, 3:22 and 4:22 p.m.

Dog 14, weight 33 kilos (pregnant).

10:20 a.m. Anesthetized with 1.32 gm. nembutal intraperitoneally, lymphatics isolated and tied, and control samples obtained as with Dog 13.

2:13 p.m. 3 cc. horse serum injected into left knee joint. This dog was kept perfectly quiet throughout. The abdomen was massaged, as well as the left leg, with care taken not to move the joint.

Collections of blood and lymph were made similarly at 2:23, 2:43, 3:13, 4:13 and 5:13 p.m.

DISCUSSION

Besides the rich subsynovial vascular supply, every joint has a similar system of lymph spaces. Magnus (11) has demonstrated that these spaces have no open connection with the joint cavity through "stomata," but are separated in every case by cells of mesenchymal origin, embedded in a matrix or ground-substance. Any passage into the lymphatic system from the interior of a joint, unless, as in the case of particles, mediated by phagocytosis, must then take place either through cells or intercellular substance. Since the lymphatic system is probably one of closed tubes (Drinker and Field, 1932), the lymphatic endothelium must also be passed in a similar manner. The last experiment described indicates that horse serum albumin readily

exercised dogs. Therefore, if one takes into account the rapidity with which a foreign protein can be removed from the blood stream, one realizes that even greater amounts have appeared in the blood stream than one detects with the precipitin tests. Such experiments further prove that our results as shown in the first experiments are not due to mere accumulation of the egg white in the blood stream.

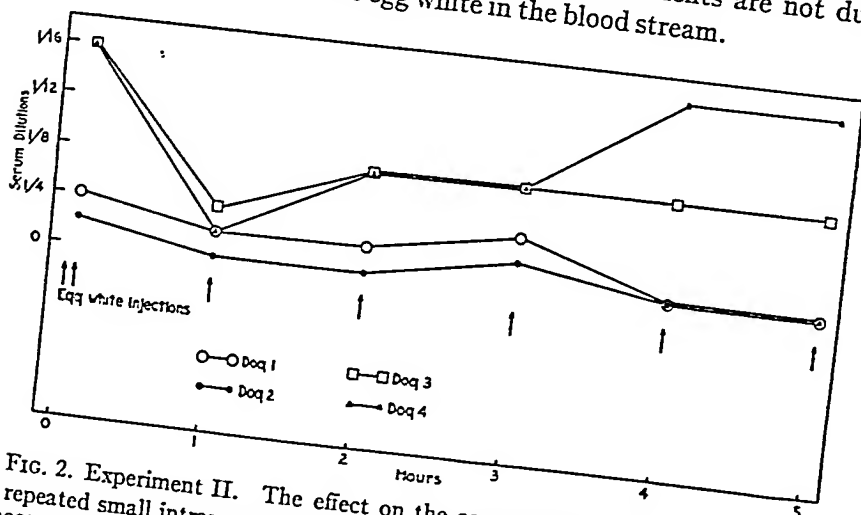


FIG. 2. Experiment II. The effect on the concentration in the blood stream of repeated small intravenous injections of egg white. Dogs 1 and 2 received 0.0027 cc. of egg white per kilo body weight, whereas Dogs 3 and 4 received 0.011 cc.

In the next series of experiments an attempt was made to determine if possible the route of removal of the injected protein.

Two dogs were used for each experiment; all the lymphatics emptying into the great vessels of the neck were tied off, thus eliminating all communications between the lymphatic and vascular systems. Cannulae in the thoracic ducts enabled us to collect frequent samples of lymph. Blood samples were taken from each dog at the same time intervals, thus allowing us to determine whether any protein can gain entrance into the blood stream if the lymphatics entering the neck veins are completely tied off. The injected knee joint of one dog was exercised as before. The abdomens of both were massaged in order to hasten the flow of lymph. The injected joint of the other dog was not touched.

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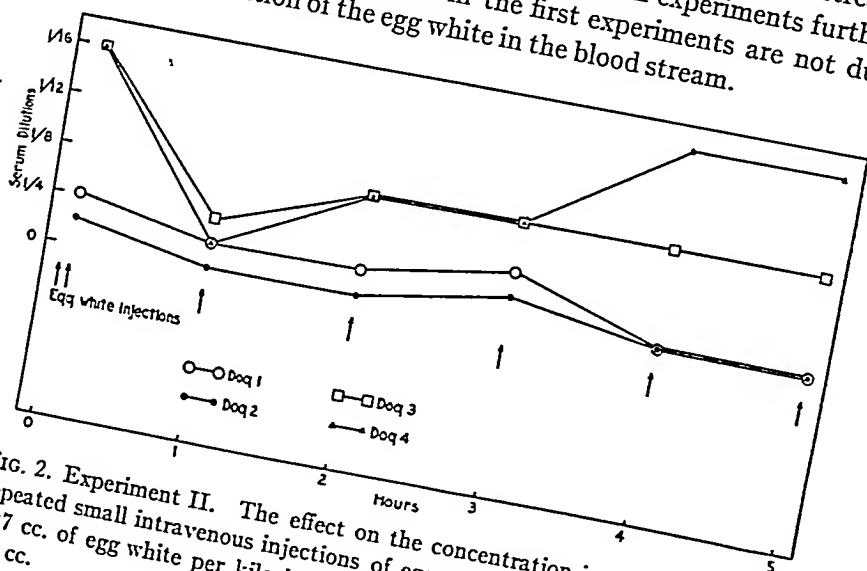


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2. Another confusing factor whose importance has been recognized only in recent years is the part played by absorptive processes in the function of the renal cells, yet the operation of such processes would make it impossible to decide how much of any observed structural change might be due to the intake or to the output of material into the urine.

3. Another almost insurmountable difficulty has been always present in that the kidney to be examined in the experiment was already in a certain functional state whose possible effects had to be distinguished from those supposedly produced by the experimental procedure. Various means were taken to put the kidney at rest before the experiment, but these could in their turn only be based on the hypothetical assumption as to what normally produced secretory activity. Moreover, it is quite possible that some of these procedures, such as fasting, may have produced structural alterations in themselves, with a resulting further confusion in the experimental results.

4. The experiments of the past were all conducted on the living animal and in many cases considerable time elapsed during their course. It will be easily recognized that with such a responsible organ as the kidney the animal itself acts as a sort of independent and not necessarily cooperative agent in the procedure and the part it plays may well defeat the entire purpose of the experiment since the investigator is easily deceived by a change in its conditions that have thus arisen unknown to him.

5. Dependence in all the investigations of the past has been placed almost solely on morphological evidence. Granules were found in rows near rods and therefore were assumed to have arisen from the rods. It is obvious that the only accurate statement of such a conclusion is that it looks as if they might have done so.

That the writers believe these severe criticisms of previous investigations have arisen from the unsatisfactory state of knowledge of the times, will be evident when it is pointed out that all of them may be directed toward certain of the investigations of the senior author (3). But very considerable advances have been made, especially those which have come from the introduction of new methods, notably direct visual examination of the kidney as devised by Richards (6) and perfusion as adapted to the frog's kidney by Hoeber (7). The investigator is now able to again examine the problem in a new light.

The following pages describe an attempt made with what may be considered another new method. This method combines a morphological and a physiological examination of the activity of the isolated perfused kidney, using histological methods for the former purpose and a modification of Hoeber's method for the latter. As a result both the structural and functional aspects of the response of the tissues to

The results of a companion experiment are shown in Fig. 4. This experiment was carried out exactly as the preceding one, the abdomens massaged, etc., except that the leg of the unexercised dog was massaged without moving the joint. Again no egg white could be detected in the blood stream of either dog. It will be seen that the curve representing the appearance of egg white and its concentration in the tho-

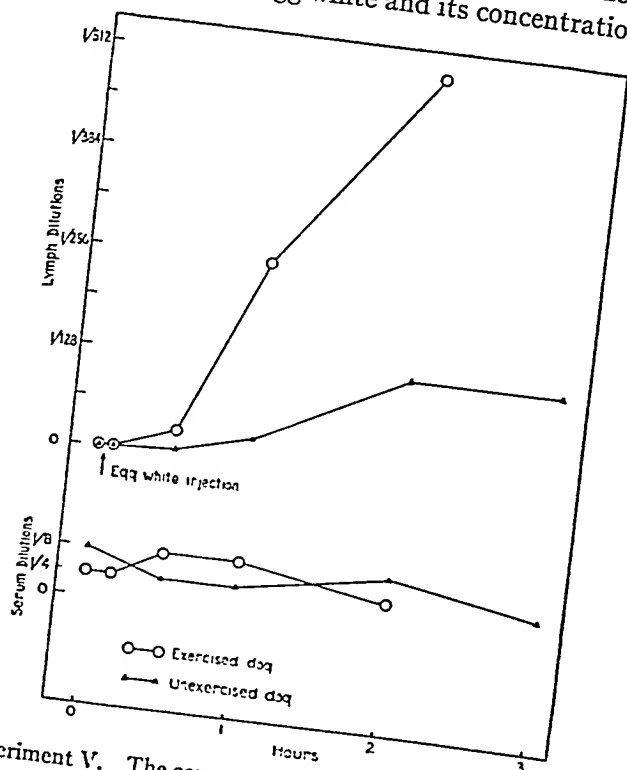


FIG. 4. Experiment V. The concentration of egg white in the blood stream and thoracic duct lymph following injection into the knee joint of 3 cc. In this case, the leg of the unexercised dog was massaged.

racic duct lymph in the exercised dog is almost an exact duplicate of that obtained in the exercised dog of the preceding experiment. However, in the case of the unexercised dog in which the leg was constantly massaged, the egg white appeared in the lymph later and did not reach as high a concentration (1/128 at the end of the 2nd hour). It must be remembered that these figures represent the appearance

Bouin's and 10 per cent neutral formalin in 0.9 per cent salt solution were used and as a stain Delafield's hematoxylin and eosin. For the preservation of the mitochondria and other granular structures of the cells Kolster's method of fixation was found most satisfactory. Bensley's fluid in our experience was less certain in its results. For staining the mitochondria and granules a method which is essentially the original Altmann procedure was used; this was supplemented by the Bensley method. For special purposes the Gram-Weigert stain was also used on tissues fixed by all the methods described above, and formol-Zenker fixation for the permanent fixation of neutral red in the tissues and subsequent embedding in paraffin and preparation of permanent sections (12).

Beside the common methods of fixation and staining other morphological procedures were used. The simplest of these was the examination of the fresh tissues. Small bits of tissue from the kidney were teased on a slide in Locke's solution and then crushed between cover-glass and slide with a twisting motion. Another valuable procedure in the morphological examination of certain problems was supravital staining with neutral red and Janus green. Vital staining of the kidneys with neutral red was also employed. The details of the use of these dyes will be given later.

Another method of staining the living tissue may be termed "extravital staining." The dye, either neutral red or Janus green, was added to the perfusion fluid after the normal function of the kidneys had been established and the cells of the tubules then stained themselves while actually functioning, a form of staining which can be called neither supravital nor vital staining.

Material from all the various vitally stained tissues was examined in the fresh condition as described above. Preparations from all were also fixed in formol-Zenker's and Kolster's fluid and permanent sections made from paraffin-embedded tissues.

The Perfusion Method.—The details of the method of perfusion of the isolated kidneys have been previously given (9). It is based on Hoeber's (7) modification of the Barkan, Broemser and Hahn (13) technique by which isotonic Locke's solution containing 0.025 per cent sugar and a small amount of glycol maintained at a pH of 7.5 is perfused through the kidneys from separate containers by both the renal arteries and the renal-portal veins. The urine is collected from each kidney in a cannula. The urine formed by the procedure, when successful, is sugar-free, its electrolyte content is less than one-half that of the perfusion fluid and the rate of its excretion is comparable to that of the formation of urine by the living frog. If urea or dyes, such as phenol red or neutral red, are added to the perfusion fluid they are concentrated in the urine. The methods of determination of the constituents of the urine in the experimentation to be described were as follows: Benedict's method for sugar, electrolyte content by the Christiansen ionometer, the results being expressed as an equivalent per cent of NaCl, and dye content by the usual colorimetric methods.

As a basis for the consideration of the experimental findings we must first describe the appearances that are found in the functioning renal

the water outside the membranes gave no test for ammonia with Nessler's reagent, the solutions were placed in sterile containers with a little chloroform and kept in the ice box. Several of the fractions showed bacterial contamination which was removed by means of a Seitz filter.

Rabbits were immunized against the three fractions, as well as against whole horse serum according to the method previously used. As expected, precipitin tests after immunization failed to show entire specificity. The two globulin fractions demonstrated complete and equal cross-immunization. In the actual experiment, therefore, immune sera only against pseudoglobulin, albumin and horse serum were used. Titration of these against the antigens used in immunization gave the results shown in Table I.

From that table, it can be seen that the separation between pseudoglobulin and albumin was incomplete, with the former containing enough albumin finally to

TABLE I

Immune sera	Dilution of antigens positive (with protein content in basic solutions in gm. per 100 cc.)		
	Horse serum 7.13 gm.	Pseudoglobulin 1.61 gm.	Albumin 0.31 gm.
1. Against horse serum	1/20,000	1/1,000	1/100
2. " pseudoglobulin	1/20,000	1/1,000	1/100
3. " albumin	1/20,000	1/10	1/1,000

immunize the rabbit slightly against it. We may assume the albumin fraction nearly free from pseudoglobulin, however, with the slightly positive reaction (1/10) gained with the albumin immunized rabbit serum explained on the albumin contamination of the pseudoglobulin fraction used as antigen.

As shown in the protocol (Experiment VI), this experiment was carried out like the two preceding ones, with horse serum containing 7.13 gm. of protein per 100 cc. injected into the knee joint of dogs prepared by tying off the lymphatics as they enter the veins of the neck; then control blood and lymph samples were taken. Again one knee joint was exercised and one kept quiet, although the leg was massaged. Precipitin tests were done on samples of blood and lymph removed at intervals, using the three immune sera described above.

Fig. 5 shows the relative appearance in the thoracic duct lymph of the two fractions. For the sake of clarity, the reactions with the rabbit serum immune to horse serum are omitted, but are contained in the complete table in the protocol and serve to confirm the data on the chart. The first point which the chart clearly demonstrates is the rapidity and ease of the removal of the albumin fraction from the

the protoplasmic structures is seen only in Segment II of the tubule. The difference in the appearance of these cells is due to a change in the relative amounts of filamentous and granular material present in the cells. Our problem now is to connect if possible this structural variation with some variation in the functional activity of the cells.

The first step in such an attempt is to determine if the changes are concomitant with some functional state. Two such states must be considered; first, absorptive activity, which includes the absorption of the normal constituents of the glomerular filtrate, such as water, sugar and salt; and secondly, secretion, as may be evidenced by the elimination of neutral red by the cells.

The Absence of Effect of Certain Absorptive Processes upon the Filamentous and Granular Material of the Cell

It is possible by means of the extravital method to cause a kidney to function so that no secretory processes are involved in its activity though absorptive processes are actively at work. If the kidney is perfused with a Locke's solution which contains only simple salts and sugar, such a condition is established, for abundant evidence has shown that these substances are eliminated as a filtrate from the plasma by the glomeruli in about the same concentration as exists in the plasma or perfusion fluid.

A frog was pithed and prepared for perfusion in the usual way except that the left kidney was removed in such a manner as to leave all the cut vessels ligated. It was then fixed in the usual reagents. The other kidney was now perfused for 1½ hours with Locke's solution. Table I shows the functional findings. It will be noticed that from the urine about two-thirds of the total salt content and all of the sugar of the perfusion fluid had been removed. Other tests would have shown that water as well had been absorbed.

When the histological appearance of the right perfused kidney by the Kolster-Altmann method was compared to that of the left or unperfused organ, no differences in the granulofilamentous material were found. In the broad Segment II of both the unperfused (Figs. 4 a, 4 b) and perfused (Figs. 5 a, 5 b) organ the cells were filled with long tortuous filaments which extended from the basal membrane to the apex of the cell. Few granular bodies could be found. The cells of the Segment IV were filled with their short rodlets in sections from both kidneys (Fig. 5 b). The neck of the tubule and the glomeruli showed no significant differences except for the presence of red blood cells in the capillary loops of the tufts of the unperfused kidney.

cause of the equal cross-immunization of the two globulin fractions, this lack of egress from the joint applies also to the euglobulin fraction of the horse serum injected. We can say, then, at this point, without

Results of Precipitin Reactions

Immune serum used and time after injection	Dilutions positive			
	Serum		Lymph	
	Unexercised	Exercised	Unexercised	Exercised
<i>Anti-horse serum</i>				
Control				
10 min.	1/128			
30 "	1/256	1/128		
1 hr.	1/128	1/128	1/128	1/32
2 hrs.	1/128	1/128	1/128	1/32
3 "	1/128	1/128	1/128	1/64
<i>Anti-globulin</i>	1/256	1/128	1/256	1/128
Control		1/128	1/256	1/64
10 min.	1/256		1/256	1/64
30 "	1/256	1/256	1/128	
1 hr.	1/256	1/256	1/256	1/128
2 hrs.	1/256	1/256	1/256	1/256
3 "	1/256	1/256	1/256	1/256
<i>Anti-albumin</i>	1/256	1/256	1/256	1/128
Control		1/256	1/256	1/128
10 min.	Negative		1/256	1/128
30 "	"	Negative		
1 hr.	"	"	Negative	Negative
2 hrs.	"	"	"	"
3 "	"	"	1/8	1/2,048
			1/2,048	1/2,048
			1/2,048	1/2,048
				1/2,048

The results obtained with the serum immune against horse serum also demonstrate that these proteins are removed through the lymphatics and not the blood stream, and more rapidly from exercised joints. The fact that no higher concentrations were obtained is probably because the serum used proved to be weak against albumin, the protein readily removed, and strong against globulin, which apparently comes out with great difficulty, if at all.

reservation, that the albumin fraction of horse serum is removed readily from a normal dog's knee joint and is detectable in high dilutions of the thoracic duct lymph under the experimental conditions employed, while the globulin fractions are removed from a normal joint with

fixed in the usual solutions, and neutral red in a concentration of 1.25 mg. per 100 cc. was added to the Locke's solution in the bottle supplying the tubular apparatus of the right kidney through the renal-portal venous system. There began an elimination of the dye which reached a rate of 1.0 mg. per hour in the third period. The right kidney was removed after two more periods and fixed. Sections of each kidney were stained by the Altmann method. The contrasting pictures are shown in Figs. 6 *a*, 6 *b* and 7 *a*, 7 *b*.

The left kidney which had been perfused with clear Locke's solution, and which had therefore no material to secrete, showed the filamentous appearance in the cells of the broad Segment II that has been previously described. Long tortuous threads filled the protoplasm and only very rarely could a definitely round granular object be found (Figs. 6 *a*, 6 *b*). The remaining divisions of the tubules showed the appearance that has been previously given.

TABLE II
Secretion of Neutral Red by the Kidney

Time	Arterial flow	Venous flow	Urine volume	Neutral red	Salt in perfusion fluid	Sugar
	<i>cc. per hr.</i>	<i>cc. per hr.</i>	<i>cc. per hr.</i>	<i>mg. per hr.</i>	<i>per cent</i>	
10:45-11:00	400	600	6.0	—	40	0
Left kidney removed						
1.25 mg. neutral red in 100 cc. Locke's solution to tubules of remaining kidney						
11:30-11:45	440	500	7.2*	0.63*	40	0
11:45-12:00	560	500	8.0	1.00	40	0
12:00-12:15	440	500	6.8	0.85	40	0
12:15-12:30	440	600	6.4	0.72	45	0

* Calculated as output of two kidneys

An entirely different picture was found, however, in the kidney which had been perfused with neutral red and which had secreted this substance into the urine. The dye by gross examination was seen to have evenly permeated the organ and sections of it showed a marked change from the appearances noted in the non-secreting kidney. In every cell of practically every Segment II no filaments could be found whatsoever, for the protoplasmic body was now distended with large round granules. In many instances they were crowded so closely together as to leave no intervening substance visible between them and so closely surrounded the nucleus as to obscure it (Figs. 7 *a*, 7 *b*). Yet in those sections where this granular appearance in Segment II was most extreme the cells of Division IV were still filled by their short thick rodlets (Figs. 8 *a*, 8 *b*). The portions of the neck and of Division III contiguous to Segment II showed a greater or lesser similarity to it.

As in the previous series of experiments with clear Locke's solution so in the tests in which neutral red was secreted, animals were found whose left kidney, perfused only by the dye-free fluid, showed a greater or lesser degree of the granu-

escapes from the interior of normal joints into the subsynovial tissues in the neighborhood of the lymphatic capillaries and is soon detectable in the lymph, whereas the globulin of horse serum does not escape readily from the interior of normal joints into the thoracic duct lymph. This type of experiment demonstrates that the size of a molecule which can be readily removed from a normal joint lies between that of horse serum albumin and horse serum globulin or between a molecular weight of 72,000 and 175,000 (Adair and Robinson (13)). Incidentally, the relative sizes of these two molecules are thus confirmed by *in vivo* experiments.

Lymphatic absorption from the peritoneal cavity is hastened by increased intraabdominal pressure (Bolton (5), Florey (14), Lemon (15)) and from the tissue spaces by massage (Lewis (4), Florey (14)). Similarly von Mosengeil (16) and Fisher (1) have demonstrated the value of joint exercise in absorption from the articular cavities. The experiments described above confirm the value, not only of exercise, but also of massage of the limb in furthering absorption of proteins. With the methods used, it is impossible to decide at present whether speeding up of lymphatic passage by massage and motion of the limb or actual changes in the intraarticular pressure, demonstrated by Smith and Campbell (17) during joint flexion, is the important factor. The conclusion that the lymphatic system is the essential removal apparatus of the joint for protein seems justified by the experimental data presented. Any interference with this drainage, as by inflammatory changes of the synovial membrane and subsynovial tissues, should be an important factor in the production and maintenance of a joint effusion. Further work is in progress to study protein absorption when various types of inflammation have been produced in the synovial membrane. It is hoped, also, to find clinical application in the prognosis and treatment of joint effusions. Certainly it would seem that a simple test could be devised, enabling us to state whether or not the lymphatic drainage of a single diseased joint is interfered with. Such a test would allow for further study of the factors involved in the production and maintenance of joint effusions. We are at present attempting to work out a simple method of carrying out such a clinical test.

The Changes Produced in the Isolated Organ by Extravital Staining with Neutral Red

Since an important part of the present study is a comparison of the findings by different methods of staining, material from the same experiments described previously, in which the appearance demonstrated by the use of the Altmann method has been noted, was also examined as examples of extravital staining.

In the experiment of Table II the remaining kidney at the end of the perfusion was found to be evenly stained by the neutral red which it had been secreting. The organ was a deep mahogany red in color, striped by the faintly pinkish bands of its connective tissue and vessels. It was not swollen nor edematous. Small bits taken from various portions of the organ were teased apart and crushed with a drop of clear Locke's solution under a cover-glass. In such specimens the dye was contained in its greatest amount in the cells of Segment II. From here the distribution continued, depending on the degree of staining, towards the neck of the tubule and downwards towards Segment IV with its rodlet cells. The former might contain a considerable amount of dye; the latter was free except for such diffuse staining as may be seen in any tissue that has been long in contact with a dye. The glomeruli contained no dye except for an occasional isolated cell of the clasmatocyte group within the tuft whose irregular protoplasm was filled with dye vacuoles.

The dye, in whatever epithelial cells were examined, was in the form of round granules. In slightly filled cells these were scattered indiscriminately through the protoplasm but in densely filled cells so packed the protoplasmic body as to completely obscure its detail. The nucleus lay as a clear round or oval area in the center of these clusters of granules and was unstained (Figs. 11, 12).

The dye may also be studied in paraffin sections from tissues fixed in formol-Zenker's and the same observations made.

The term "granule" has been used to describe the round deeply staining structures chiefly because that term seemed most appropriate for the description of appearances in the fixed tissues stained by the Altmann method. To most, the word "granule" holds the connotation of solidity and the term "vacuole" might therefore seem more appropriate for the appearances noted in the extravital preparation. That they are not vacuoles in the sense that they are cavities in the protoplasm filled with free liquid dye is shown, however, by the fact that when the cell is crushed they remain intact and float through the surrounding Locke's solution in which the cell is suspended. They remain discrete indefinitely and show little tendency to coalesce, their appearance being that of small droplets of red-stained semifluid material. For we may be sure that they are not solid substance by means of a change similar to one described in vitally stained animals by Policard (2) that occurs in them as the preparation stands. This consists in the

CELLULAR MECHANISMS OF RENAL SECRETION.
A STUDY BY THE EXTRAVITAL METHOD

I. THE STRUCTURAL PHASE OF THE SECRETORY MECHANISM*

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Hoagland Laboratory, Brooklyn)

PLATES 28 TO 31

(Received for publication, October 24, 1932)

It is evident from a survey of the voluminous literature of renal activity that a state of confusion exists in our knowledge concerning the secretory activity of the renal cells. In illustration of this fact we may call attention to an example of the conflict in conclusions and theories that prevails and examine briefly the reasons for its existence.

If one considers the relatively simple problem of the correlation between morphological alterations in the cells, specifically in the mitochondrial apparatus, and functional activity, a great discrepancy of opinion is found, ranging from the belief of such investigators as Kolster (1), Policard (2) and Oliver (3) that such changes may be correlated with varying functional states, to that of others, such as Cushny (4) and Emge (5) who are skeptical of any relation whatsoever between these two aspects of cell activity. General opinion is therefore quite properly unsettled.

Certain reasons for this condition are not far to seek in the light of present knowledge.

1. The basic difficulty has been that we have had no exact definitive knowledge of what part, if any, secretion may play in the elimination of substances into urine. Experiments have had to be designed on a hypothetical basis and have therefore proceeded not from the known but from the assumed. As an example of this, an increase in the volume of urine due to the intravenous injection of salt solution has been assumed to produce an increase of secretory activity. It is not surprising that the sought for structural changes in the renal epithelium were indefinite, when one considers that we know now that the increased urine eliminated under such conditions is best accounted for by the activity of other parts of the renal unit than the tubule.

* This investigation has been made with the assistance of a grant from the Josiah Macy, Jr., Foundation.

cell, clustering around the nucleus where they appear as short bacillary or vibrio-like objects. In neutral red extravital preparations they are never seen, for here all the stained objects are the definitely round large granulovacuoles. That these thread-like structures are indeed present and that they too may be stained extravitally is shown, however, in the next series of experiments.

The Changes Produced in the Isolated Organ by Extravital Staining with Janus Green

The method of extravital staining with Janus green was identical with that used with neutral red. The perfusion was begun with clear Locke's solution and after one or two samples were examined to determine if a normal condition of kidney

TABLE III
Action of Janus Green on the Kidney

Time	Arterial flow	Venous flow	Urine volume	Dye	Salt in perfusion fluid	Sugar
	<i>cc. per hr.</i>	<i>cc. per hr.</i>	<i>cc. per hr.</i>	<i>mg. per hr.</i>	<i>per cent</i>	
10:00-10:15	400	500	6.0	—	45	0
1/500,000 Janus green in Locke's to tubules of remaining kidney						
10:30-10:45	400	450	6.0	0	45	0
10:45-11:00	450	400	8.0	0	40	0
11:00-11:15	500	400	10.0	0	45	0
11:15-11:30	450	450	7.2	0	45	Tr.

function had been established, the dye was added to the fluid in the bottle that supplied the tubular circulation. Janus green is a definitely toxic substance, so that a dilution of 1/500,000 was used. Another advantage of this dilution whose importance will be emphasized later is the specificity of its staining reaction. But even with such a solution the kidneys showed evidences of damage after 2 or 3 hours of perfusion so that the definitive experiments were limited to 1 hour or less.

The results of such extravital staining with Janus green are shown in Table III. It will be observed that there was no serious disturbance in the function of the organ, but the dye was not excreted. An explanation of this lack of secretion will be considered later.

In the gross, the kidneys were unchanged except for their dark slate-green color. When examined in fresh crushed specimens many of the cells of Segment II appeared similar, save for color, to those from the neutral red kidney. Other cells contained the large green round granulovacuoles but when examined with the oil immersion a striking difference from the earlier preparation with neutral

either physiological or pathological stimuli can be observed and correlated simultaneously. The application of the method to some of the problems of experimental nephritis has already been described and the term "extravital" suggested to describe it (8).

Another aid to our immediate problem has been found in the discovery that the dye neutral red is eliminated by the isolated perfused kidney almost entirely through the tubular epithelium and not in any significant amount through the glomeruli (9). Similar findings had been reported independently by Scheminzy (10) and recently the occurrence of its tubular elimination has been supported by the findings of Richards (11) that in the living frog the degree of its glomerular filtration is not adequate to account for its appearance in the urine.

The possible use of the extravital method in the problem under discussion for a study of the definitely proven secretion of neutral red is evident. By means of such a combination we can be certain that the tubule cells of a kidney eliminating neutral red are actually secreting, and we can also be certain that the dye found in the cell bodies has come from the blood vessel and not been absorbed from the lumen of the tubule. Absorptive process alone may also be produced by the new method in the functioning organ without any secretory activity, and the histological pictures then compared. The extravital method also avoids a further difficulty we have mentioned since the state of the kidneys before the experiment can be determined either by examination of one kidney before the perfusion is started or a portion of the kidney that is to be perfused may be examined before or at any stage of the experiment. So, too, any modification of the activity of the kidney on the part of the animal is prevented by the isolation of the organ. The nervous system is eliminated and all of the constituents of the fluid with which the cells are working are accurately known and these can be modified as desired as the experiment proceeds. The urine is collected almost simultaneously with the activity of the cells, rather than in long periods after its formation. And, finally, it is possible to examine the problem, placing little weight on a subjective interpretation of morphological evidence.

Technique

Histological Methods.—The following methods have been used for the morphological part of the work. For a general picture of the state of the tissues Zenker's,

taining the Janus green solution that had been supplying the tubules was now replaced by one containing Locke's solution with neutral red in a concentration of 1.25 mg. in 100 cc. and the perfusion was continued for three more 15 minute periods with no serious impairment of any function save that the elimination of neutral red was markedly depressed. The significance of this striking difference from the profuse elimination of neutral red in the previous experiments can be discussed more clearly after the results of the experiments of the following paper have been described. The organs were now examined and appeared grossly normal except for their muddy brownish red-green color.

TABLE IV

Action of Janus Green and Neutral Red on the Kidney

Time	Arterial flow	Venous flow	Urine volume	Dye	Salt in perfusion fluid	Sugar
	cc. per hr.	cc. per hr.	cc. per hr.	mg. per hr.	per cent	
10:45-11:00	400	760	4.0	—	40	0
1/400,000 Janus green in Locke's to tubules						
11:00-11:15	400	760	4.8	0	45	0
11:15-11:30	400	800	3.6	0	40	0
11:30-11:45	360	760	3.6	0	50	Ft. tr.
11:45-12:00	400	760	3.2	0	45	Ft. tr.
1.25 mg. of neutral red per 100 cc. Locke's to tubules						
12:00-12:15	400	740	4.8	Tr. n. red		Ft. tr.
12:15-12:30	400	700	4.0	Tr. n. red	45	—
12:30-12:45	400	760	4.4	Tr. n. red	40	Ft. tr.

A low power examination of fresh crushed specimens showed at once that there had occurred a mixed staining of the tissues with the two dyes. In some of Division II the cells were uniformly filled with either green or with red granules and in the former the bacillary mitochondria could be seen. In other tubules there was a mixed staining of individual cells. In some were red and green droplets in varying proportions indiscriminately scattered throughout the cell body. In such cases each droplet seemed to be stained purely with one or the other dye (Fig. 14). Others though showing pure red and pure green granules also contained some of these bodies definitely stained with a mixture of the two dyes which gave them a purplish slate-green color (Fig. 15). With all such appearances the fine green mitochondria might be found. Even the picture shown in Fig. 16 was observed where in a protoplasm filled with many green mitochondria only a few large neutral red droplets were present.

The varied pictures seen in these preparations have but one interpretation; namely, that the granulovacuolar bodies which were

cells of the living frog. The description that follows is essentially the same as that of Policard (2).

The Histological Characteristics of the Renal Cells of the Living Frog

Since the epithelial cells vary in their structure in different segments of the tubule the appearances seen in each are given separately.

1. The neck, or narrow portion I, of the tubule is lined with a low layer of cuboidal cells from which arise long actively motile flagella. The nucleus is small and round. The protoplasm contains a moderate number of granules which stain heavily with the acid fuchsin of the Altmann mixture. Very occasionally short thread-like structures may be seen among the round granules.

2. Segment II of the tubule is lined with a much thicker epithelium whose cells are covered with a brush border and whose nuclei are round or slightly oval. Many different histological pictures are presented by the protoplasm of the cell. It may be filled with long tortuous filamentous structures often so closely packed together that little free protoplasm is seen, which run from its base to within a short distance of its apex. In such cells no round granules are found (Figs. 1 a, 1 b). In contrast to the filamentous appearances just described the cell may show none of these structures, its entire protoplasm being crowded with large round granules that completely fill and even distend the cell body (Figs. 2 a, 2 b).

Between these two extremes all degrees of combination are possible. The general picture in the two kidneys of an animal is the same, though it may vary in different tubules and even between cells in the same cross-section of a tubule.

3. Segment III of the tubule connects the broad Segment II, or proximal convoluted tubule, with Segment IV or distal convoluted tubule. Its epithelium presents no distinctive characteristics but shows a gradual transition from the type described for Segment II to that which will be described for Segment IV.

4. Segment IV, or distal convoluted tubule, is lined with cuboidal cells with oval nuclei and which possess no brush border. Their protoplasm is distinguished by the presence of short thick rodlets which run almost the entire length of the cell. Granules are rarely found among its protoplasmic constituents (Figs. 3 a, 3 b). That the filaments and granules of Segment II as well as the rodlets of Segment IV are preexisting structures and not the result of the action of the fixative solution may be easily shown by the examination of fresh tissues from the kidney in Locke's solution. The large round granules appear as highly refractile droplet-like objects while the filaments, though much less clearly seen than in the fixed material, are easily discernible as delicate striations or as an indefinite strippling of the protoplasm. The rodlets of Segment IV are more clearly outlined as stout refractile rodlets.

The findings in normal frogs living under physiological conditions may be summarized as follows: Marked variation in the condition of

course impossible to perform elaborate tests with these less controllable methods. Enough of the salient features of the previous experiments can be observed with them however to afford conviction that the processes that occur in the handling of the dye are similar under the conditions that obtain in all three methods, vital, supravital and extravital. The findings that we give are in a summary form.

Appearance of the Supravitality Stained Renal Epithelium

Supravital Staining with Neutral Red.—Bits of fresh kidney tissue from killed animals were teased and agitated in a concentration of 0.25 mg. neutral red in 100 cc. Locke's solution. At intervals small portions were removed and examined in fresh crushed smears with Locke's solution. The appearances in the cells can be summarized by the statement that they are identical with those we have described as a result of the extravital method. A few minutes after being placed in the dye solution red granules appeared in the cells of Segment II of the tubule which increased in number with time, so that in a short period, whose length depended apparently chiefly on the speed with which the dye could permeate the teased tissues, the protoplasm of the cells was filled with large round droplets that surrounded the unstained nucleus (Fig. 18). There was no staining of the mitochondria nor were the rodlets of Division IV colored. This latter portion of the tubule was also free of the larger round granulovacuoles. In the vacuoles, especially in the older preparations, the same small motile granule within the larger one was seen. In other words one cannot distinguish a fresh crushed specimen of such a preparation under the microscope from one from the previously described extravitality stained kidney.

Supravital Staining with Janus Green.—Bits of kidney tissue placed in Locke's solution containing a 1/500,000 concentration of Janus green showed in the course of a few minutes dye within the protoplasm of their cells. The poor penetration, however, of Janus green as compared to neutral red was particularly noticeable in the supravital preparations. Only the cells of the tubules on the periphery of the tissue masses were stained and these irregularly. The protoplasm of the cells of Segment II was filled with the fine bacillary mitochondria and among these were scattered large round green droplets in greater or less number. As time passed the latter increased in number while the mitochondria decreased (Fig. 19). In the vacuoles there developed the small motile granule and the rodlets of Division IV of the tubule were stained a definite green. In specimens that had stood for some time evidences of damage appeared. These consisted of irregularities in size and shape in both the mitochondria and the droplets and a greenish discoloration of the nucleus associated with pycnosis.

Combined supravital staining with the two dyes was also done and the appearances noted were entirely the same as in the combined extravital experiment. The double staining was accomplished by staining the tissues in mixtures of the

This experiment shows the typical findings in kidneys whose granulofilamentous material of Segment II was in a filamentous state before the perfusion began. In repetitions of the experiment various conditions were found in the kidney removed before perfusion. In some there were no filamentous structures at all in the cells of Segment II, and these were filled and distended with large round granules. Yet the rodlets of Segment II were still visible and these cells contained few or no granules. In other experiments combinations of filaments and granules in varying amount were seen in Segment II before perfusion. In all these cases the unperfused kidney showed exactly the same arrangement of the stainable material, whether filamentous or granular, in the cells of Segment II.

TABLE I
Absorptive Processes Only in the Kidney

Time	Arterial flow	Venous flow	Urine volume	Salt in perfusion fluid	Sugar
	<i>cc. per hr.</i>	<i>cc. per hr.</i>	<i>cc. per hr.</i>	<i>per cent</i>	
10:45-11:00	680	880	3.6*	27	0
11:00-11:15	720	800	5.6	39	0
11:15-11:30	760	840	4.8	38	0
11:30-11:45	760	840	6.0	40	0
11:45-12:00	720	800	5.2	38	0

* Calculated as output of two kidneys.

It is reasonable to infer two important facts from these experiments. First, that the method of perfusion does not in itself affect the histological appearance of the filamentous granular material. And second, that the processes of absorption, in as far at least as they concern the important urinary constituents of salts, water and sugar, can be eliminated as a possible cause of any morphological changes that may be found in the granulofilamentous structures of the cells of Segment II.

The Effect of a Secretory Process on the Granulofilamentous Material of the Renal Cells

The evidence which indicates that neutral red is secreted by the tubule cells of the kidney has been previously mentioned (9-11). In the following experiment the effect of this secretory process on the protoplasmic constituents of these cells is demonstrated.

The frog was prepared for perfusion and a normal urine was formed as shown in table II. After one sample had been collected the left kidney was removed and

extravital experiments. It follows that in the present instances the results of vital, extra- and supravital staining were essentially similar in their nature.

DISCUSSION

A summary can now be made of the structural changes that accompany secretion in the cells of Segment II and their significance can be appraised.

The following facts have been established.

1. Filaments and granulovacuoles are preformed protoplasmic elements of the renal cell.

2. During the normal life of the animal the cells of Segment II show a wide variation in the relative amounts of these two protoplasmic structures. In any given cell there is a converse relation between the amount of filamentous and granular material.

3. The principal absorptive processes that occur in the formation of the urine are not accompanied by variations in these structures.

4. The extravital secretion of neutral red which has been shown to be identical in its processes with vital secretion, is accompanied by a disappearance of filaments and the formation of granulovacuoles. If the cells originally contain only the latter, no histological alteration accompanies the secretion of the dye.

5. The granulovacuolar structures stain with neutral red during the secretion of the dye. This can be demonstrated either extravitally or vitally.

6. The filamentous structures never stain with neutral red.

7. The filaments do stain with Janus green. They also require bichromatization for their preservation and are soluble in weak acetic acid.

8. The granulovacuolar material also stains with Janus green even from weak concentrations of the dye but does not require bichromatization and is not soluble in dilute acetic acid.

9. The filaments are Gram-negative, the granulovacuolar structures Gram-positive.

That the structural changes observed as concomitant with the secretion of the dye play a part in the actual secretory process seems certain when one considers that the dye is concentrated within the cell in the

lar state in Segment II of its tubule instead of the purely filamentous appearance. The functional activity of these kidneys under the perfusion differed in no way from that of the preceding experiment. The appearances in Segment II of the non-secreting and secreting kidney were, however, found less dissimilar than those described in the preceding experiment. If the cells of the first kidney, non-secreting, were filled with granules and contained no filaments, then the second, secreting, was identical in appearance. If, however, some filaments were present in the first kidney, a dissimilarity was found, for the cells of the second kidney which had secreted the dye contained none of these structures but were filled with granules alone. As an example, the appearance of Segment II is shown in Figs. 9 *a*, 9 *b*, 10 *a*, 10 *b* and 10 *c*.

The experiments with neutral red just described allow the definite conclusion that in contrast with the negative effect of absorptive processes there occurs concomitantly with the secretion of neutral red a change in the histological appearance of the cells of Segment II that is characterized by the disappearance of filaments and the appearance of granules. If the cells are already in the granular form before the neutral red is given, then no further change occurs in the protoplasmic structures with the secretion of the dye. Secretion of the dye is therefore accompanied by structural changes in the renal cells that are identical with those noted as a part of the vital activities of the living animal and secretory activity occurs only with the histological picture of granule formation.

With the evidence at hand we are justified in stating that the change from the filamentous to the granular state is concomitant with secretory activity. Can a more intimate relation between the morphological and functional aspects of cell response be demonstrated so that we can definitely conclude that the functional activity is determined by the structural change? There remain also several questions of detail. Of the protoplasmic structures of the renal cell, what is mitochondrial substance and what is not? Are the granules and filaments independent structures or are they related in their constituent material or by a common origin? Are there various sorts of "granules" involved in the histological picture? Are the granules solid substance or are they of the liquid nature and therefore best considered vacuoles? All these questions will be examined directly and objectively by the method of extravital staining and the findings compared with those obtained by the other vital staining and fixed tissue methods.

plex of the mitochondrial material has occurred, with a consequent liberation of its constituent substances, so that each, now no longer bound and inert, reacts in its characteristic manner. The freed lipoid takes up neutral red while the protein element, insoluble in alcohol without bichromatization, reacts with the Gram stain like fibrin.

One might visualize the course of events in the secretory process already described somewhat as follows. Neutral red enters the cell from the blood vessel. Owing to its presence and to factors as yet undetermined the mitochondrial filaments disintegrate. One can think of many forces that might cause such a result, such as changes produced by the presence of the dye in interface surface tensions, osmotic pressure or diffusion currents. From the material of the disintegrated filaments and as a result of constituent alterations, vacuoles are formed in which the dye being readily soluble is concentrated. Whatever vacuoles may be present from previous processes of secretion are also saturated with the dye. And here the description of the process of elimination must, for the time being, end, for no evidence whatsoever has been obtained as to how the dye passes from the vacuoles into the lumen of the tubule. This question will be examined in the succeeding article and the relation of our findings to those of previous observers will be discussed.

CONCLUSIONS

1. The secretion of neutral red reproduces those variations which are observed in the mitochondrial apparatus of the renal tubule cells of animals living under native conditions. The tubular absorptive processes concerned with water, salts and sugars do not produce these effects.

2. The changes in the mitochondria consist of both structural and constituent alterations. These have been shown to be not merely phenomena concomitant with secretion, but a determining factor in one part of this process; namely, in the concentration of the dye within the cell.

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development within the granule of a smaller granule, a minute point-like or comma-shaped object which has all the appearance of condensed dye that has precipitated out of the dye content of the larger granule. The smaller object is usually affected by Brownian movement and may be seen twisting and turning within the larger granule, colliding with its inner surface and rebounding into the center again. This active movement proves that the substance of the granule proper must be at least semifluid, so, with our previous determination that its substance is immiscible with water and from what we know of the solubility of neutral red, the assumption that the granule is a droplet of lipid material becomes extremely plausible. In one sense, therefore, the structure is a vacuole but in order to maintain the concept that fits the appearance in the fixed tissues so well and which has been used almost entirely by those who have previously studied such material we shall use the terms, "granule," "vacuole" and "droplet" as synonyms.

The question now presents itself as to the exact relation of these extravitally stained vacuoles and the large round granules which have been described in the Altmann preparation of fixed tissue from the extravitally stained kidney. Since we know from the examination of fresh tissues that the granules of the fixed tissues are preexisting objects a glance at Figs. 7a, 7b and 11, 12, which are from the same kidney stained by the two methods, will show that the extravitally stained objects and the Altmann-stained granules are certainly the same formations. Apart from the similarity of their appearance there would not be space for two preexisting different sets of such structures to exist side by side within the limited room of the cell body. The granule of the extravitally stained kidney can therefore be considered a semifluid lipid droplet deeply stained with neutral red which when fixed is preserved and stainable by the fuchsin of the Altmann method. It would seem reasonable to extend this conclusion to include in the same category the unstained granules seen in cells of the living animal which resemble identically by the Altmann method the objects seen in the extravitally stained neutral red experiment. Supporting evidence of this identity will appear later.

In the description of the kidney stained extravitally with neutral red there has been no mention of filaments within the cells. It is true that these structures have disappeared, at least in their long thread-like form, with the occurrence of secretory activity and the development of the granules but, as previously described for the Altmann preparations, portions of them always remain in the lower part of the

plex of the mitochondrial material has occurred, with a consequent liberation of its constituent substances, so that each, now no longer bound and inert, reacts in its characteristic manner. The freed lipid takes up neutral red while the protein element, insoluble in alcohol without bichromatization, reacts with the Gram stain like fibrin.

One might visualize the course of events in the secretory process already described somewhat as follows. Neutral red enters the cell from the blood vessel. Owing to its presence and to factors as yet undetermined the mitochondrial filaments disintegrate. One can think of many forces that might cause such a result, such as changes produced by the presence of the dye in interface surface tensions, osmotic pressure or diffusion currents. From the material of the disintegrated filaments and as a result of constituent alterations, vacuoles are formed in which the dye being readily soluble is concentrated. Whatever vacuoles may be present from previous processes of secretion are also saturated with the dye. And here the description of the process of elimination must, for the time being, end, for no evidence whatsoever has been obtained as to how the dye passes from the vacuoles into the lumen of the tubule. This question will be examined in the succeeding article and the relation of our findings to those of previous observers will be discussed.

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1. The secretion of neutral red reproduces those variations which are observed in the mitochondrial apparatus of the renal tubule cells of animals living under native conditions. The tubular absorptive processes concerned with water, salts and sugars do not produce these effects.

2. The changes in the mitochondria consist of both structural and constituent alterations. These have been shown to be not merely phenomena concomitant with secretion, but a determining factor in one part of this process; namely, in the concentration of the dye within the cell.

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red was noted. For besides the large round green granules there were found in those parts of the cell where the protoplasm was not occupied by them fine thread-like dark green bacillary objects (Fig. 13). Another difference from the neutral red staining was noted in Segment IV. In the Janus green extravital preparations the short thick rodlets were definitely stained distinct green. There were, however no large green vacuolar formations in this division of the tubule.

The staining of the thread-like bodies with Janus green together with their reaction with bichromate and their appearance when stained by the Altmann method leaves no doubt that these objects can be considered mitochondria in the strict and modern sense of the word. The same is true for the rodlets of Division IV. That these filamentous mitochondria have disappeared in great part from the cell bodies of Segment II of kidneys actively secreting neutral red and that they only persist in those portions of the protoplasm that are not occupied by the large droplet formations is worthy of especial note.

The nature of the large droplets in the Janus green extravital preparations is less certain, however, for they have all the appearance, save color, of the objects seen in the kidney stained extravitally with neutral red, and mitochondria do not stain with this dye. Our next problem must be, therefore, to determine whether these structures in the Janus green preparations are indeed the same objects seen in the neutral red preparations.

All the morphological similarities we have mentioned are strong presumptive evidence that the large round green bodies of Janus green extravital staining are identical to the red ones of neutral red extravital staining. Still one might demur to such a conclusion with the suggestion that in the former case the droplets have arisen as a result of some specific or toxic action of the Janus green. The extravital method allows us, however, to answer this objection in a decisive manner for it can be shown conclusively that the two appearances are the same object stained by one or the other dye.

The Changes Produced in the Isolated Kidney by Combined Extravital Staining with Janus Green and Neutral Red

Since neutral red is a more rapidly acting dye than Janus green the latter was perfused first to the tubule of the kidney in a dilution of 1/400,000 in the manner previously described. Table IV shows the results. For 1 hour a urine normal except for the appearance of faint traces of sugar was obtained. The bottle con-

plex of the mitochondrial material has occurred, with a consequent liberation of its constituent substances, so that each, now no longer bound and inert, reacts in its characteristic manner. The freed lipid takes up neutral red while the protein element, insoluble in alcohol without bichromatization, reacts with the Gram stain like fibrin.

One might visualize the course of events in the secretory process already described somewhat as follows. Neutral red enters the cell from the blood vessel. Owing to its presence and to factors as yet undetermined the mitochondrial filaments disintegrate. One can think of many forces that might cause such a result, such as changes produced by the presence of the dye in interface surface tensions, osmotic pressure or diffusion currents. From the material of the disintegrated filaments and as a result of constituent alterations, vacuoles are formed in which the dye being readily soluble is concentrated. Whatever vacuoles may be present from previous processes of secretion are also saturated with the dye. And here the description of the process of elimination must, for the time being, end, for no evidence whatsoever has been obtained as to how the dye passes from the vacuoles into the lumen of the tubule. This question will be examined in the succeeding article and the relation of our findings to those of previous observers will be discussed.

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stained indiscriminately by either Janus green or neutral red or by both in mixture are in fact the same structures. It is a fact of considerable significance as will appear later that these structures stain so readily in a very dilute solution of Janus green.

Our experiments have demonstrated one definite difference in the reaction of the mitochondria and the granulovacuolar bodies, since the former never stain with neutral red. Another reaction has been found which indicates a difference in the constitution, either physical or chemical, of the two structures, and that is their reaction to the Gram stain. In all specimens, whether from animals living under natural conditions, from kidneys perfused with plain Locke's or from those extravitally stained with either neutral red or Janus green, the granulovacuolar bodies retain the Gram stain (Figs. 23-25). Such Gram positivity, however, is never present in the mitochondria, either in the fine bacillary forms and long filaments of Segment II or in the short rodlets of Segment IV (Fig. 26).

Another difference between the vacuoles and the mitochondria that can be demonstrated by means of the Gram-Weigert stain is the fact that the former do not need to be bichromatized to resist solution in alcohol as do the mitochondria, and also that they resist the action of acetic acid in the fixing solution, a substance which completely destroys all evidence of mitochondrial structures (Figs. 23, 24).

Again the question may arise if we are dealing with the same granulovacuoles in these sections stained with Gram as in the preparations stained by the extravital method.

A Gram stain done on tissues after such treatment in which the neutral red has been preserved by fixation in formol-Zenker's solution shows cells containing blue vacuoles, red vacuoles, a mixture of the two and granules whose color is a purplish combination of red and blue (Fig. 17).

As stated, it is our plan to bring to bear as many different methods as are applicable to the problem in order to confirm our contention that the extravital method not only produces no artifacts in the tissues that are being examined by it, but that under its conditions processes of the same nature may develop as actually occur during life. For these reasons supravital and vital staining with the two dyes was done and the results compared with those of extravital staining. It is of

amount. The filaments are replaced by large granulovacuoles and no filaments are visible. Figs. 8 *a* and 8 *b* show the unaltered rodlets of Segment IV from the same kidney.

FIGS. 9 *a*, 9 *b*, 10 *a*, 10 *b* and 10 *c*. Effect of the secretion of neutral red on cells of Segment II which originally contained a mixture of filaments and granules. Experiment of page 443. Kolster-Altmann procedure. Figs. 9 *a* and 9 *b* show the condition of the cells of Segment II of the kidney removed before the secretion of neutral red. Many filaments and a certain number of granules are seen. Figs. 10 *a* and 10 *b* show similar cells from the other kidney which had been perfused with and which had secreted neutral red. The filaments have entirely disappeared and the cells are filled with large round granules. Fig. 10 *c* shows the rodlets of Segment IV unchanged.

PLATE 30

All figures, except No. 17, are of fresh preparations of unfixed tissue suspended in Locke's solution.

FIG. 11. Extravital staining with neutral red. Experiment of Table I. A portion of Segment II whose cells have retained their normal position in the tubule. They are filled with granulovacuoles of neutral red. The nuclei are unstained. Magnification $\times 390$.

FIG. 12. Extravital staining with neutral red. An isolated cell from Segment II of the same kidney. Clustered around the nucleus are granulovacuoles of neutral red. Compare the appearance with that of the same tissues after the Kolster-Altmann procedure (Figs. 7*a* and 7*b*) and after Bouin fixation and Gram-Weigert staining (Fig. 23) and Zenker fixation and Gram-Weigert staining (Fig. 24). Magnification $\times 1200$.

FIG. 13. Extravital staining with Janus green. Unfixed cell from Segment II of the kidney of the experiment of Table III. The large granulovacuoles of Janus green are seen as well as the fine bacillary thread-like mitochondria. Note that in this and the following figures mitochondria occur only in parts of the cell free of granulovacuoles. Magnification $\times 1200$.

FIG. 14. Extravital staining with both neutral red and Janus green. Experiment of Table IV. There is an indiscriminate mixing of red and green granules throughout the protoplasm of the cell with a few scattered mitochondria. Magnification $\times 1200$.

FIG. 15. The same specimen and procedure. Another cell from Segment II shows red and green vacuoles as well as some which are stained with a mixture of the two dyes. This mixture is represented for purposes of reproduction as a stipple whereas in fact the admixture of dyes produces an even greenish brown tone. Magnification $\times 1900$.

FIG. 16. The same specimen and procedure. Many fine bacillary forms of mitochondria stained with Janus green are visible. All of the granulovacuolar bodies are stained with neutral red. Magnification $\times 1200$.

vacuolar structures during the course of the secretion. The significance of this concentration is examined in an accompanying paper. That the filaments are mitochondrial is also obvious. The question remaining for determination is that of the nature of the vacuoles and of what relation they bear to the mitochondria. From a consideration of the facts noted above, two interpretations are possible.

1. The filaments and granules are entirely independent structures that bear no relation to each other. On this hypothesis the mitochondria play no rôle in the secretion of the dye. But if such is assumed one is at once confronted with the difficult admission that highly diluted Janus green is not so specific a stain as a long series of investigations would indicate. There also remains without explanation the mysterious disappearance during the secretory process of the filaments which one must assume have vanished leaving no trace while at the same time an equally unexplained appearance *de novo* of granulo-vacuoles has occurred, derived not from the mitochondria, but from an unknown substance which has the generally recognized characteristic staining reaction of mitochondria.

2. The granulovacuoles are derived from the mitochondrial filaments by alteration both in their form and constituent substance. The change in substance is of such nature that neutral red now stains them, leaving unaffected, however, the original staining reaction of this altered mitochondrial material to Janus green.

The second interpretation seems to us much the more satisfactory. It explains, for example, the converse occurrence of filaments and granules which is so constantly observed; the reaction of the vacuole to dilute Janus green as a remainder of the characteristics of the original mitochondrial substance; it indicates what becomes of the substance of the disappearing mitochondria and designates from what source the material of the originating granulovacuoles is derived. By it the mitochondria are made essential factors in the secretion of the dye, since the change which occurs in them is the source of the final mechanism by which the dye is concentrated within the cell body. The exact nature of this alteration which accompanies the change of the mitochondrial substance into vacuolar substance is not definitely shown by the experiments but the altered reactions of the latter to dyes and fixatives suggests that a splitting of the protein-lipoid com-

vacuoles. The same specimen with the Altmann stain is shown in Figs. 2 *a*, 2 *b*. Magnification $\times 525$.

FIG. 26. Segment II of another normal untreated animal. Kolster fixation, Gram-Weigert stain. No Gram-positive granules are visible, as the cells in this instance are filled with mitochondrial filaments. Compare Figs. 1 *a* and 1 *b*, a section from the same block stained by the Altmann method. Magnification $\times 525$.

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EXPLANATION OF PLATES

PLATE 28

Magnification of photographs $\times 525$; of drawings $\times 680$.

FIGS. 1 *a* to 3 *a*, and 1 *b* to 3 *b*, tubules from two animals living under native conditions, Kolster-Altmann procedure. Figs. 1 *a* and 1 *b* show Segment II of the tubule. The cells are filled with long filamentous structures. Figs. 2 *a* and 2 *b* show Segment II from another animal. Its cells are greatly swollen and filled with large round granules. Figs. 3 *a* and 3 *b*, from the same specimen as Nos. 2 *a* and 2 *b*, show sections of Segment IV. The flat cells are filled with heavy rodlets. They contain no granules.

FIGS. 4 *a*, 4 *b* and 5 *a*, 5 *b*. The negative effect of absorptive processes on the filaments of Segment II. Experiment of Table I. Kolster-Altmann procedure. Figs. 4 *a* and 4 *b* show the cells of the kidney removed before the perfusion was started, filled with long filaments and containing no large round granules. Figs. 5 *a* and 5 *b* show the other kidney of the animal after 1½ hours perfusion with plain Locke's solution. Under these conditions absorption by the tubule cells of water, salts and sugar occurred, but no secretion of any substances. The cells show no alteration from their previous condition. In the right part of Fig. 5 *b* Segment IV is seen with its unaltered rodlets.

PLATE 29

Magnification of photographs $\times 525$; of drawings $\times 680$.

FIGS. 6 *a*, 6 *b*, 7 *a*, 7 *b*, 8 *a* and 8 *b*. The changes produced by the secretion of neutral red on the filaments of Segment II. Experiment of Table II. Kolster-Altmann procedure. Figs. 6 *a* and 6 *b* show the cells of Segment II of the kidney removed after the preliminary perfusion with plain Locke's solution that contained no secretable substances. They are filled with long filaments and contain no granules. Figs. 7 *a* and 7 *b* show Segment II from the other kidney that was perfused with neutral red and which secreted this substance in considerable

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EXPLANATION OF PLATES

PLATE 28

Magnification of photographs $\times 525$; of drawings $\times 680$.

Figs. 1 *a* to 3 *a*, and 1 *b* to 3 *b*, tubules from two animals living under native conditions, Kolster-Altmann procedure. Figs. 1 *a* and 1 *b* show Segment II of the tubule. The cells are filled with long filamentous structures. Figs. 2 *a* and 2 *b* show Segment II from another animal. Its cells are greatly swollen and filled with large round granules. Figs. 3 *a* and 3 *b*, from the same specimen as Nos. 2 *a* and 2 *b*, show sections of Segment IV. The flat cells are filled with heavy rodlets. They contain no granules.

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PLATE 29

Magnification of photographs $\times 525$; of drawings $\times 680$.

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4. Cushny, A. R., The secretion of the urine, London, Longmans, Green and Co., 2nd edition, 1926.
5. Emge, L. A., *Stanford Univ. Pub., Univ. Series, Med. Sc., No. 2*, 1921, 1.
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7. Hoeber, R., *Klin-therap. Woch.*, 1927, 6, 671.
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concentration in the cell we shall begin with this feature of the secretory process.

The Determination of the Concentration of Neutral Red in the Tissues of the Secreting Kidney

Experiments were performed in the manner described in the foregoing paper. Neutral red was perfused through the venous circulation of the tubules while clear Locke's passed through the glomerular circulation. The urine was collected as usual in 15 minute periods and the dye content determined. After from 3 to 5 hours, the experiment was stopped. The kidneys were then removed intact, freed of all adventitious tissues, dried by repeated blotting until no more

TABLE I

Concentration of Neutral Red in the Kidney Tissue from a Solution of Locke's Containing 1.25 Mg. per 100 Cc.

Average rate of secretion of neutral red over long period experiment	Neutral red in kidney tissue	Concentration factor <small>Kidney tissue Perfusion fluid</small>
<i>mg. per hr.</i>	<i>mg. per 100 gm.</i>	
0.03	411	328
0.03	354	283
0.09	782	626
0.10	939	751
0.25	2250	1800
0.30	420	336
0.50	261	208
0.60	419	335
0.80	446	332

fluid came from their dull surfaces and weighed. Each was then ground with weak acid alcohol in a mortar until only a few bits of colorless fibrous tissue remained. After centrifugalization the dye content in mg. per 100 gm. of kidney substance was determined colorimetrically and the concentration in the kidney tissue as compared to that of the perfusion fluid calculated. Table I shows some of the results. It will be seen that there were considerable variations in the concentration of dye in the tissues. These may be explained by the varying length of time of the perfusion and the efficacy of it which depends in turn on the flow through the vessels. This flow varies considerably due to irregularities in vascular tone. We need not, however, consider the cause of variations in the concentrating process here but shall confine ourselves to points that immediately concern the problem of the output of the dye. That the concentration in the cells is not directly concerned with this essential phase of secretion is shown by the fact that

FIG. 17. Extravital staining with neutral red, fixation in formol-Zenker's solution and counterstaining by the Gram-Weigert method. Experiment of Table I. A cell from Segment II. Some of the granules have retained their neutral red color, some are heavily stained by the methyl violet, represented as black, while others show a mixture of the two dyes. These latter present an even purplish red tone that has been represented by a coarse stipple. Magnification $\times 1900$.

FIG. 18. Supravital staining with neutral red. A cell from Segment II of the kidney of an untreated animal supravitaly stained. The protoplasm is filled with deeply stained red granulovacuoles, and the picture is identical to that obtained by extravital staining. Compare Fig. 12. Magnification $\times 1200$.

FIG. 19. Supravital staining with Janus green. A similar cell from the same kidney illustrated in Fig. 18, supravitaly stained. Many green granulovacuolar bodies and a few fine mitochondria are visible. Compare with Fig. 13, an extravitaly stained cell. Magnification $\times 1200$.

FIG. 20. Supravital staining with both neutral red and Janus green. Cell of Segment II from same material supravitaly stained in Locke's solution containing $1/100,000$ neutral red and $1/500,000$ Janus green. A mixture of red and green vacuoles is clustered about the nucleus. For similarity to the results of combined extravital staining of the two dyes compare with Fig. 14. Magnification $\times 1200$.

FIG. 21. Supravital staining with neutral red and Janus green. Same material and method. Fine green mitochondria are visible with areas of clustered neutral red granulovacuolar bodies. The corresponding appearance by the combined extravital method is seen in Fig. 16. Magnification $\times 1200$.

FIG. 22. Vital staining with neutral red, with subsequent supravital staining with Janus green. A cell from Segment II of the kidney of an animal vitally stained with neutral red. Material from this kidney was then supravitaly stained in Locke's solution containing $1/500,000$ Janus green. The vital granulovacuolar bodies of neutral red alternate with areas where the persisting mitochondria have been supravitaly stained by the Janus green. The effect is identical with combined extravital (Fig. 16) and combined supravital staining (Fig. 21). Magnification $\times 1200$.

PLATE 31

FIG. 23. Segment II of the tubule from the kidney secreting neutral red in experiment of Table II. Bouin fixation, Gram-Weigert stain and no counterstain. The cells are filled with granulovacuoles that have resisted the action of acetic acid and retained methyl violet. Compare with Figs. 7 *a* and 7 *b* where the same structures are stained by the Kolster-Altmann procedure. Magnification $\times 525$.

FIG. 24. From the same kidney. Zenker fixation, Gram-Weigert stain. The same Gram-positive granules fill the cells. Magnification $\times 525$.

FIG. 25. Segment II of a normal animal living under native conditions. Kolster fixation, Gram-Weigert stain. The cells are filled with Gram-positive granulo-

in the last period when the cells were obtaining dye from the vessels is taken as 100 per cent and the amount excreted in the latter periods when the cells were receiving no dye is expressed as a percentage of this amount. It will be seen that there was a rapid and progressive fall in the amount excreted which, after three or four periods, reached a fairly constant level at about 5 per cent of the previous secretion. The first sample studied, collected 15 minutes after the change, cannot be considered to indicate the amount of dye obtained from the concentrating vacuoles alone, for immediately after the change to clear fluid was made there must have been a certain amount of dye free within the cells in the process of elimination and this was obtained in the first and perhaps the second sample collected. The importance of this fact will be referred to later.

TABLE II

The Contribution to the Urine of the Dye Concentrated in the Kidneys

Time	Arterial flow	Venous flow	Urine volume	Dye	Salt in perfusion fluid	Sugar
	cc. per hr.	cc. per hr.	cc. per hr.	mg. per hr.	per cent	
Tubules perfused with 1.25 mg. per 100 cc. Locke's solution						
11:00-11:15	320	400	5.2	0.34	40	0
11:15-11:30	320	400	6.0	0.32	40	0
11:30-11:45	320	320	7.2	0.20	40	0
Clear Locke's to tubules of kidneys						
11:45-12:00	320	320	6.0	0.08	—	0
12:00-12:15	320	400	5.2	0.04	45	—
12:15-12:30	240	320	4.4	0.02	—	0
12:30-12:45	240	320	4.0	0.01	—	—
12:45-1:00	240	400	4.0	0.01	45	0

Concentration of dye in kidneys = 424 mg. per 100 gm. = 336 times concentration in original perfusion fluid.

A confirmation of the small part played by the dye concentrated within the vacuoles in the actual output of dye into the lumen can be obtained in another way as the following experiment shows.

The kidneys of an animal were perfused as described above with neutral red Locke's solution and the urine collected, the samples from each kidney being kept separate. After three 15 minute periods had passed, the left kidney was removed with ligation of all the cut vessels and without disturbing the right kidney. The bottle of dye-containing Locke's that was now perfusing only the right kidney was replaced with clear Locke's and the collections continued from this one kidney until a minimum amount of dye was excreted. The right kidney was now removed and the concentration of dye in the two organs determined. A comparison shows that



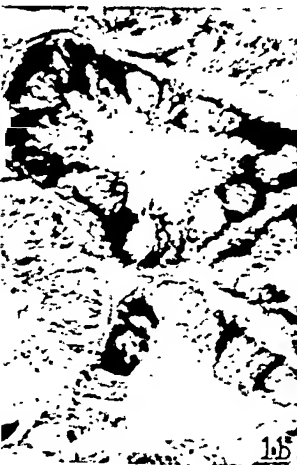
1a



2a



3a



1b



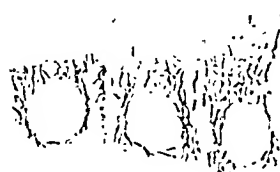
2b



3b



4a



5a



4b



5b

10. (a) and (b) Cellular components of plant tissue. 11

from the kidneys in these experiments did not pass into the urine but back into the Locke's solution in the blood vessels. As this fluid was collected in the outflow bottle from the vena cava after passing through the kidneys it was definitely stained with dye. That such a back flow from kidney tissue to blood vessels may occur in the living animal has been suspected and, in the case of urea, actually demonstrated (1).

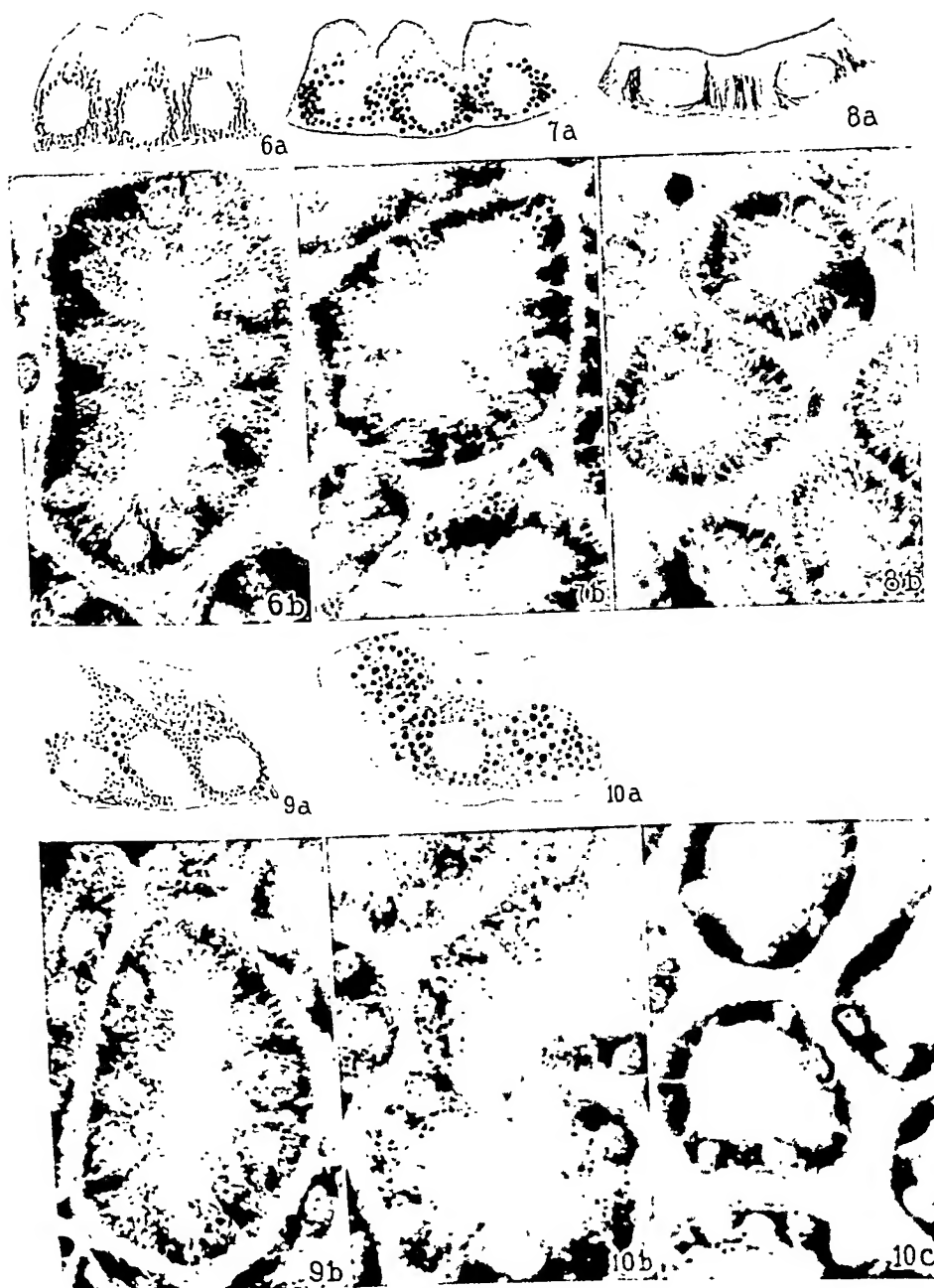
The conclusion to be drawn from these experiments is that only a small amount, probably $\frac{1}{10}$ or $\frac{1}{20}$, of the dye that appears in the urine at the height of secretion under ordinary conditions can be derived from the granulovacuolar mechanisms.¹ The bulk of the dye present in the urine must have entered the cells from the blood vessels and passed through them to be discharged directly into the lumen of the tubules independently of these structures. The question may therefore be asked as to how much of the dye that enters the cell is concentrated in the granulovacuolar apparatus and thus withheld for a certain time from elimination and how much is directly discharged into the lumen of the tubule.

A Factor That Determines the Processes of Concentration and of Elimination

It can be shown that one factor which influences the distribution of the dye between concentration in the vacuoles and direct elimination into the lumen is the concentration of it in the blood stream. The following experiment illustrates this point.

The kidneys of an animal were perfused through the venous system with Locke's solution containing a low concentration of neutral red of 0.3 mg. per 100 cc. The procedure was identical with that of the experiment illustrated in Table II. Table III shows the result of a series of such experiments. The next group of experiments was done in exactly the same way except that the dye in the solution going

¹ The objection may be raised that under the conditions of the experiments just described the failure of the dye to pass from the vacuoles into the urine is due to a lack of passage of it into the vacuoles from the blood stream; that a continuous intake into the vacuole is necessary for its output. But our first experiments have shown that, with a continuous supply and even with an increasing concentration in the vacuoles, elimination nevertheless falls, a fact to be emphasized by other experiments that follow (Table V). So too it will be shown that the passage of the dye from a concentrated phase to one less concentrated is not dependent on any such continuing supply of dye to the concentrated phase (page 475).



10, Ver and Lund: Cellular mechanisms of renal secretion. 11

under vital conditions for processes analogous to those just described. It is common knowledge that neutral red appears in the urine shortly after its injection into the blood stream of an animal and that it is concentrated in the kidney epithelium for some time, conditions which are essentially those described above as occurring in extravital kidneys. We have tried, however, to obtain some quantitative data in regard to the relations involved in the living animal.

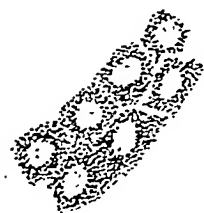
A series of frogs were stained vitally by the repeated injection of 0.25 per cent solution of neutral red into their dorsal lymph sacs. After three injections the animals were anesthetized with urethane and a portion of the left kidney removed. All bleeding was stopped by ligature, the wound was closed by sutures and the animals replaced in their tank. They recovered from the anesthetic and seemed entirely normal. The concentration of neutral red in the portion of kidney re-

TABLE IV
Concentration of Dye in Kidneys of Living Animal

Original dye in kidney	Dye in kidney after 20 hrs.	Per cent remaining
<i>mg. per 100 gm.</i>	<i>mg. per 100 gm.</i>	
43.8	2.4	5.4
36.1	5.6	15.5
30.0	5.6	18.0
25.7	1.8	7.0
16.6	7.2	43.0

moved was now determined as previously described. 20 hours later the animals were killed and the dye content remaining in their kidneys determined. The results are shown in Table IV. Although there is considerable variation it will be noted that after 20 hours the slow elimination of the dye which had been concentrated in the kidney was continuing.

It is apparent from the results of the experiments done so far that the secretion of neutral red by the renal cells is not a single unified procedure but that its elimination must be divided into two very different processes which are independent to a large degree of each other. The first type of secretory process occurs only when there is a considerable concentration of the dye in the blood vessel, it occurs promptly and is of considerable amount. The second is a slow long continued elimination which may proceed after the dye has completely



11



12



13



14



15



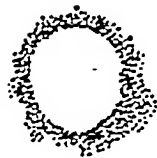
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22

Table V shows the flow through the vessels of the two systems and the elimination of water, salts and dye. It will be observed that there was a marked decrease in the secretion of the latter though the rate of salt and water secretion remained approximately constant.² In this experiment the cause of the decrease in the secretion must be a local cellular one for the rate of flow through the blood vessels did not significantly vary and the concentration of dye in them was constant. The spinal cord had been destroyed so that nervous effects were at a minimum.

Is this local effect on some specific phase of the direct passage of the dye through the cells into the lumen and if so can we more accurately locate the site of the changes that produce it? The next experiment answers this question.

TABLE VI
Effect of Bichromate on Direct Secretion

Time	Arterial flow	Venous flow	Urine volume	Dye	Salt in perfusion fluid	Sugar
	<i>cc. per hr.</i>	<i>cc. per hr.</i>	<i>cc. per hr.</i>	<i>mg. per hr.</i>	<i>per cent</i>	
10:15-10:30	520	800	6.4	—	35	0
10:30-10:35	15 cc. potassium bichromate to tubules					
10:45-11:00	520	600	5.7	—	41	Tr.
11:00-11:15	560	800	9.6	—	50	+
	1.25 mg. neutral red in 100 cc. Locke's solution to tubules					
11:15-11:30	560	800	12.0	0.039	52	+
11:30-11:45	520	800	10.4	0.040	50	+
11:45-12:00	520	800	10.4	0.030	52	+

Concentration of dye in kidneys 782 mg. per 100 gm. = 626 times concentration in perfusion fluid.

A Specific Alteration in Direct Secretion as a Result of Inner Membrane Damage without Impairment of the Concentrating Processes of Indirect Secretion

The kidneys were perfused as usual with clear Locke's solution passing to both the tubules and the glomeruli. After urine formation was normally established,

² Such a gradual fall in the elimination of neutral red is almost constantly observed in any perfusion of the isolated kidney. It explains, for example, the rather marked differences in the rate of elimination of the dye given in Tables III and I. In the former the figures represent the average of the optimal direct secretion as found in the first periods of a perfusion, the latter the average of the rates of elimination in longer experiments in which direct secretion had to a greater or less degree fallen off.

CELLULAR MECHANISMS OF RENAL SECRETION. A STUDY BY THE EXTRAVITAL METHOD

II. THE FUNCTIONAL PHASE OF THE SECRETORY MECHANISM*

By JEAN R. OLIVER, M.D., AND EDNA MORRIS LUND

(From the Department of Pathology of the Long Island College of Medicine, The
Hoagland Laboratory, Brooklyn)

PLATE 32

(Received for publication, October 24, 1932)

Although the morphological aspects of the secretory process have allowed us to form some idea as to what occurs when the dye enters the cell body from the blood stream and have shown us the mechanism by which it is concentrated there, nothing has been learned from such data as to the final definitive act of secretion; that is, the discharge of the dye into the tubule lumen. The dye was left in our description concentrated in vacuoles. But these vacuoles neither moved through the cell nor did they burst and liberate their content into the lumen nor were they discharged into it as such. It is true that such mechanical methods of renal cell discharge have been described by isolated investigators but the great majority of workers have denied their occurrence. Certainly the newer methods we have used would have disclosed such gross phenomena if they had been present. *A priori* it is therefore certain that since the discharge of the dye is not associated with structural change its mechanisms must be investigated by other than morphological methods. And here another advantage of the extravital method, combining as it does in the same experiment the two aspects of cell activity, is evident, for the same experiments that we have used can be also employed in obtaining evidence concerning the less obvious phenomena of the final act of secretion.

Since we have already examined the morphological evidence of

* This investigation has been made with the assistance of a grant from the Josiah Macy, Jr., Foundation.

nation from its original figure has occurred during the course of a normal perfusion experiment. The findings of Gellhorn (4) and Sato (3) suggest that this decrease in passage of the dye might possibly be overcome by increasing the concentration of K ions in the Locke's solution or by decreasing the Ca ions. The following experiment examines this point.

The kidneys were perfused in the usual way, with ordinary Locke's solution containing neutral red in a concentration of 1.25 mg. in 100 cc. passing to the tubules

TABLE VII
Effect of K and Ca Ions on Direct Secretion

Time	Arterial flow	Venous flow	Urine volume	Dye	Salt in perfusion fluid	Sugar
	cc. per hr.	cc. per hr.	cc. per hr.	mg. per hr.	per cent	
1.25 mg. of neutral red in 100 cc. Locke's solution to tubules, KCl = 0.01 per cent, CaCl ₂ = 0.02 per cent						
10:10-10:15	400	840	7.0	0.21	40	0
10:15-10:30	420	840	6.0	0.18	40	0
10:30-10:45	440	840	6.8	0.14	40	0
Neutral red as before, KCl = 0.015 per cent, CaCl ₂ = 0.01 per cent						
10:45-11:00	400	800	7.2	0.20	40	0
11:00-11:15	440	840	8.0	0.26	45	0
11:15-11:30	400	840	7.2	0.21	40	0
11:30-11:45	400	840	6.2	0.19	—	—
11:45-12:00	400	800	6.0	0.23	—	—
12:00-12:15	400	800	5.7	0.31	45	0
12:15-12:30	440	840	6.0	0.29	—	—
12:30-12:45	400	840	6.0	0.31	—	—
12:45-1:00	360	800	6.0	0.30	40	0
1:00-1:15	320	800	4.0	0.50	—	0
1:15-1:30	320	800	4.0	0.49	45	Tr.

and clear Locke's to the glomeruli. In such a solution the KCl concentration is 0.01 per cent and the CaCl₂ concentration 0.02 per cent. Table VII shows the results. It will be seen that as the perfusion continued there occurred a gradual and progressive fall in the rate of elimination of neutral red from 0.21 to 0.14 mg. per hour. When the rate of secretion of the dye had reached this last figure the fluid in the bottle supplying the tubules was changed to a Locke solution containing an increased concentration of KCl, 0.015 per cent, and a decreased concentration of CaCl₂, 0.01 per cent. The fluid contained the same amount of neutral red as previously and its pH was not significantly altered by the changes in salt concentra-

the output of dye bears no direct relation to the concentration within the cell. As much or more dye was secreted per hour with a concentration in the cell body 336 times that of the perfusion fluid as when the intracellular concentration was 1800 times. And of the same significance is the observation that with a gradually increasing concentration of dye in the tissues, a fact that may be roughly determined during the course of the experiment by gross examination of the kidney's color, there may go a decreasing rate of elimination into the urine.

The result of these findings indicates at once that the older conception of secretion whereby concentration in the cell body was assumed to be the preliminary step and determining factor in the passage of the dye into the lumen of the tubule is far too simple. Output and intracellular concentration must be considered separately. Our first step was to determine how great a part the concentrating process in the granulovacuolar structures may play in the output of dye into the urine.

The Contribution of the Concentrating Granulovacuolar Structures to the Output of Dye

In the perfused organ it is possible at the height of the secretion of the dye to suddenly change the dye-containing fluid that is coming by way of the venous system to the tubules so that the cells are at once bathed with clear Locke's which contains no dye. It is evident that under such altered conditions any dye that is secreted after the change has been made, must of necessity be derived from that which has been concentrated in the cell. This amount compared to that which was being eliminated when the cells were receiving dye from fluid in the vessels in the period before the change, will allow us to estimate what part may be contributed by the concentrating process to secretion. The experiment was performed as follows:

The kidneys were perfused in the usual manner, neutral red passing only to the tubules. After three periods of fairly constant secretion of the dye the bottle containing neutral red that was supplying the tubules was replaced by one containing the same clear Locke's as was passing to the glomeruli. The connecting tubing and cannulae were flushed free of the dye-containing Locke's so that in the course of 1 minute or less a change had been made and the tubules were receiving no dye whatsoever. The collections of urine were continued for five or six periods. The details of a typical experiment are shown in Table II and the results of several are illustrated in Text-fig. 1. In this chart the amount of neutral red excreted

dye concentrated within the vacuoles might have been liberated and thus have become available for elimination; or there may have occurred an increased permeability in the outer (vascular) membrane of the cell with a resulting entrance of more dye and hence greater passage of it through the cell. It is not necessary to discuss the probability of these possibilities, for the seat and method of action can be directly demonstrated by experimental means.

TABLE VIII

Lack of Effect of K and Ca Ions on Inner (Lumen) Membrane

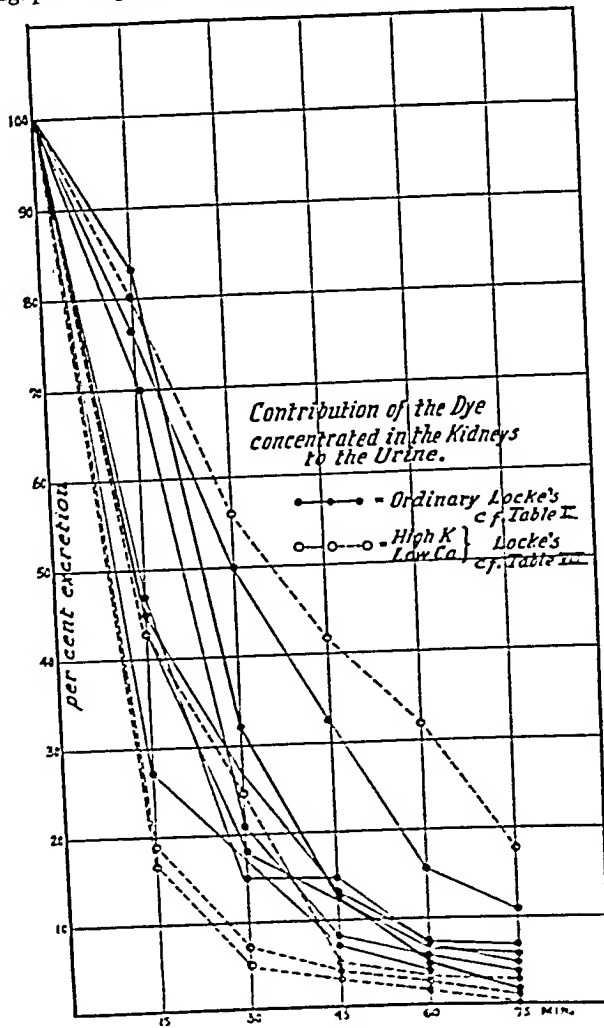
Time	Arterial flow	Venous flow	Urine volume	Dye	Salt in perfusion fluid	Sugar
	cc. per hr.	cc. per hr.	cc. per hr.	mg. per hr.	per cent	
Tubules perfused with 1.25 mg. neutral red per 100 cc. Locke's solution, KCl concentration = 0.01 per cent, CaCl_2 concentration = 0.02 per cent						
10:15-10:30	500	600	9.0	1.3	40	0
10:30-10:45	400	500	6.0	1.0	40	0
10:45-11:00	400	640	8.0	1.6	—	0
11:00-11:15	440	640	8.0	1.3	45	0
Clear Locke's to tubules, KCl concentration = 0.015 per cent, CaCl_2 concentration = 0.01 per cent						
11:15-11:30	400	600	7.6	0.56	45	0
11:30-11:45	400	600	7.6	0.07	—	—
11:45-12:00	400	640	6.4	0.05	45	0
12:00-12:15	360	640	6.5	0.01	—	—
12:15-12:30	360	640	5.8	0.01	45	0

The Site of Ion Action on the Cell Membranes

To examine this question the method of experiment previously described was used in which the intake of dye into the cells is suddenly stopped in the course of active secretion. In the present experiment, however, the perfusion after this stoppage was continued not with the ordinary Locke's but with this fluid so modified as to contain a high ratio of KCl/CaCl_2 . The results of such procedure were then compared to the earlier experiments with ordinary Locke's solution. A typical experiment was done as follows. Its results are shown in Table VIII.

The tubules of the kidney were perfused with ordinary Locke's solution containing the usual amounts of KCl in 0.01 per cent, CaCl_2 in 0.02 per cent and

though there is less dye in the right kidney than in the left, 622 mg. per 100 gm. as against 904 mg. per 100 gm., this disproportion is insignificant as compared to that



TEXT-FIG. 1

existing between the amount of the dye that the two kidneys were secreting at the moment of their removal; namely, 0.005 mg. per hour and 0.100 mg. per hour.

Another phenomenon seen in this experiment should be noted although it does not concern the secretory process. The greater part of the dye that was removed

membrane changes compared with the single process of concentration in the vacuolar structures, why the course of the direct secretion of the dye varies so markedly as compared to the relative stability in the operation of the indirect secretory process.

The extreme stability of the indirect method of secretion under adverse conditions is a remarkable fact. Kidneys that are being perfused with a solution of neutral red, though they not infrequently fail to eliminate neutral red in a normal manner, never fail to take up and concentrate the dye in their granulovacuolar system and to proceed with its slow indirect secretion. Why is the latter so markedly resistant to the effect of damage?

The Degree of Stability of the Concentrating Processes of Indirect Secretion

A reason for this marked resistance is shown in the following experiment.

Kidneys were perfused in the usual manner with clear Locke's solution passing to both glomeruli and tubules. Table IX shows the results. After normal function was established, 20 cc. of 1/1000 corrosive sublimate in Locke's solution was passed by way of the veins to the tubules. Following this, neutral red in a concentration of 1.25 mg. in 100 cc. was added to the clear Locke's solution supplying the tubules. As will be seen in the table very little dye reached the urine which was scanty in amount, high in salt and contained sugar. The perfusion was continued for six periods and the kidneys then removed. Their appearance was strikingly different from that of normally stained kidneys or from those which had been stained after small doses of potassium bichromate, for they were now a brick-reddish yellow in color and very firm in consistency. Fresh crushed specimens showed no evidence of vacuoles or any other granular or filamentous structures in the cells. The protoplasm was stained a definite yellowish hue instead of the mahogany-red seen in the normal kidneys and even the nucleus was tinged by the yellow color. The appearance of these obviously dead cells in fixed and stained sections was similar to that previously described (5) as a result of sublimate under extravital conditions (Fig. 1).

The appearance of the fresh tissue showed a definite shift towards the alkaline side in the reaction of the dye within the cell but it did not appear from either gross or microscopical examination that there had been any definite concentration of the dye above that which existed in the perfusion fluid. However, when the kidneys were weighed and the dye extracted, it was found that it was 18 times as concentrated in the dead kidney tissue as in the fluid which had bathed the cells.

to the tubules was in the higher concentration of 1.25 mg. per 100 cc. of Locke's solution. The findings are also shown in Table III.

If the ratio of dye eliminated to the concentration of it in the vessels is compared in the two cases it is seen that a higher ratio of elimination is found with a high blood concentration than with a low one. On the other hand, the relative degree of concentration in the kidney is higher when the concentration in the vessels is low. This allows us to conclude that the concentration in the blood is one factor that determines how much of the entering dye is concentrated in the granulo-

TABLE III

Effect of Concentration of Dye in Perfusion Fluid on Various Secretory Processes

Average optimal direct secretion* of neutral red	Ratio: Rate of secretion Concentration in perfusion fluid	Concentration factor in kidney tissue
Perfusion fluid = 0.3 mg. neutral red per 100 cc.		
0.018	0.06	1005
0.023	0.07	1408
0.030	0.10	1036
0.019	0.06	1561
Perfusion fluid = 1.25 mg. neutral red per 100 cc.		
0.60	0.48	335
0.80	0.64	332
1.1	0.88	208
	Low concentra- tion of neutral red	High concentra- tion of neutral red
Relative values of concentration of dye in perfusion fluid...	1	4+
Average relative values of ratios of direct secretion.....	1	9+
Average relative values of concentration factor in tissues....	3+	1

* A definition of the term direct secretion is given on page 467.

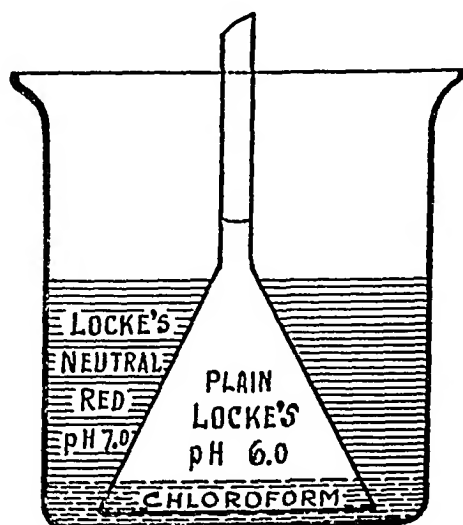
vacuolar bodies and how much is directly and immediately eliminated. Another factor which doubtlessly affects the amount of dye stored in the vacuoles is the degree of their saturation with the dye. Unfortunately we have been unable to investigate this point for reasons that will appear later.

The Demonstration of Similar Concentrating Processes during the Secretion of Neutral Red by the Living Animal

Though our previous experience with the extravital method has led us to believe that the activities occurring under its conditions are similar to those occurring in the living animal, it seems best to search

allowing a stream of CO_2 to bubble through it. Under these conditions the dye was concentrated in the chloroform layer and passed into the inner fluid.

How relatively efficient such physical models are as compared to the living cells is difficult to decide. It must be remembered that the indirect secretion of neutral red by living cells is at best a feeble process, and the great part that may be played by purely mechanical relations of the dye-containing fluid to the concentrating medium, chloroform, is shown by a simple experiment. If chloroform is shaken even gently with the alkaline dye-containing Locke's, so that it forms



TEXT-FIG. 3

isolated droplets (vacuoles) it concentrates within itself almost immediately all the dye of the fluid. If this dye-saturated chloroform is now gently shaken with acid dye-free Locke's solution the dye is almost instantly transferred to the aqueous medium. Such alterations in the physical relationships of the two phases make the model an infinitely more efficient mechanism for the transference of the neutral red from one medium to the other than are the living undamaged renal cells.

DISCUSSION

The results of the experiments described in this and the preceding studies may be considered in their general and specific aspects. The

disappeared from the blood stream and this elimination is always low in its rate. The mitochondrial granulovacuolar apparatus of the renal cell plays no observable part in the first type of secretion, but is definitely the controlling mechanism of the second. Since throughout the remainder of our discussion we must distinguish between these two processes that, combined, result in what has been called "secretion," we shall call the first, direct, and the second indirect secretion. The latter term implies the fact that the dye which ultimately reaches the lumen of the tubule has passed indirectly to it by the intermediate vacuoles.

TABLE V
Fall in Direct Secretion of Neutral Red

Time	Arterial flow	Venous flow	Urine volume	Dye	Salt in perfusion fluid	Sugar
	cc. per hr.	cc. per hr.	cc. per hr.	mg. per hr.	per cent	
Tubules perfused with 1.25 mg. neutral red per 100 cc. Locke's solution						
10:00-10:15	440	920	12	0.78	45	0
10:15-10:30	440	800	11	0.89	—	0
10:30-10:45	520	920	11	0.92	—	—
10:45-11:00	440	800	10	0.73	40	—
11:00-11:15	440	920	10	0.52	—	0
11:15-11:30	360	800	8	0.39	—	—
11:30-11:45	440	920	10	0.31	45	0
11:45-12:00	440	920	10	0.30	45	0

The mechanism by which indirect secretion operates, the mitochondrial granular apparatus, is known and, since this seems to play no part in direct secretion, our next problem is a search for the controlling mechanisms of this process. One thinks at once of membranes and changes in their permeability as controlling factors in the passage of the dye. An attempt, therefore, was made to see if such a concept may be applied to explain the variations occurring in the direct secretory process. The following experiment shows that local cellular changes must influence the output of the dye into the lumen and since these variations are rapid in their development and of considerable degree it is certain that they must be affecting the direct and not the indirect secretion of the dye.

The kidneys of an animal were perfused in the usual manner, the tubules receiving neutral red in the usual concentration of 1.25 mg. per 100 cc. Locke's solution.

into the lumen of the tubule of the dye that has entered the cell from the blood vessel. This has been termed "direct secretion." The mechanism which controls it is the variation that occurs in the permeability of the two cell membranes, the one lying adjacent to the blood vessel, the other contiguous to the lumen of the tubule. Each of these membranes may be affected independently of the other by different factors. The permeability of the outer, or vascular, membrane depends on the balance between K and Ca ions of the fluid bathing it. The permeability of the inner, or lumen, membrane may be decreased by toxic substances such as potassium bichromate, without any significant change in that of the outer membrane. Depending as it does, therefore, on the reciprocal action of the two membranes that may function in the same or in an inverse sense, direct secretion is an easily disturbed process. Such disturbances account for the remarkably complex variations in the abnormal kidney's activity that are observed with the damaged organ in the secretion of neutral red and the absorption of substances from the urine that we have described in a previous study (2).

The other method of secretion has been called the indirect. It is characterized by the concentration of the dye within the cell to as much as 3000 times that of the perfusion fluid in the vessels. The elimination is a slow long continued process that in the living animal may be observed at least 20 hours after the original entrance of the dye into the cells. The distinctive mechanism concerned is an alteration in the mitochondrial apparatus of the renal tubule cells. These changes can be observed microscopically and consist of a disappearance of the filamentous mitochondria and the development from mitochondrial substance of large granulovacuolar structures which differ in their fixative and staining reactions from the original mitochondrial material although they still retain the characteristic reaction of the latter to Janus green. One of the most striking of these differences is the Gram-retaining power of the granulovacuoles as compared to the Gram-negative quality of the filamentous mitochondria. That these changes are not simply concomitant with the concentration of the dye, or the result of it, but that they are processes which determine in part secretion is evident from the fact that one of the changes is the acquirement by the granulovacuoles of the property of staining

15 cc. of potassium bichromate in Locke's solution in a dilution of 1/10,000 was passed to the tubules through the cannulae supplying the venous system. The effect on the formation of the urine is shown in Table VI. The failure of absorptive processes is evident in the increase in water and salt and in the appearance of sugar (12). Neutral red in the usual concentration of 1.25 mg. per 100 cc. Locke's solution was now passed to the tubules and the collection of urine continued. It was observed that only very small amounts of dye were eliminated, only a faint pink grossly visible with the naked eye as contrasted to the Burgundy-red urine that appears in the first periods of an experiment from a normal kidney (compare Table V). At the close of the experiment the kidneys were removed and their dye content examined by the method previously described. A concentration 626 times that of the perfusion fluid was found, a figure well within the range of the dye content previously found in normally secreting kidneys (compare Table I).

A consideration of these results shows definitely that the action of the bichromate was not in preventing the dye entering the cell nor in any change in the cell's ability to hold it concentrated in the vacuolar apparatus. And since it did not reach the lumen in normal amount the change producing the decreased elimination must therefore have occurred at the inner cell surface adjoining the tubule lumen.³

That the effect in the above experiment was limited at least relatively to the inner (lumen) membrane has been easily demonstrated. A question somewhat more difficult to answer arises when one asks if the outer (vascular) membrane may be in turn specifically altered. The observations of Sato (3) and of Gellhorn (4) on the effect of ions on the permeability of cell membrane are suggestive. But before speculating on the possibility of ion action and its exact situation we must first demonstrate that such action affects the passage of the dye through the cells of the secreting kidney.

The Increased Passage of Dye through the Renal Cells as a Result of Ion Action

The possible action of an alteration of the ionic balance in the Locke's solution on the passage of dye through the cells may be particularly well examined in kidneys in which the not uncommon fall in dye elimi-

³ We are not here concerned with the mechanisms of the absorptive processes in the cell but the failure of absorption of water, salt and sugar is presumptive evidence that the permeability of the inner membrane is also decreased in an opposite direction to that of the secretory process.

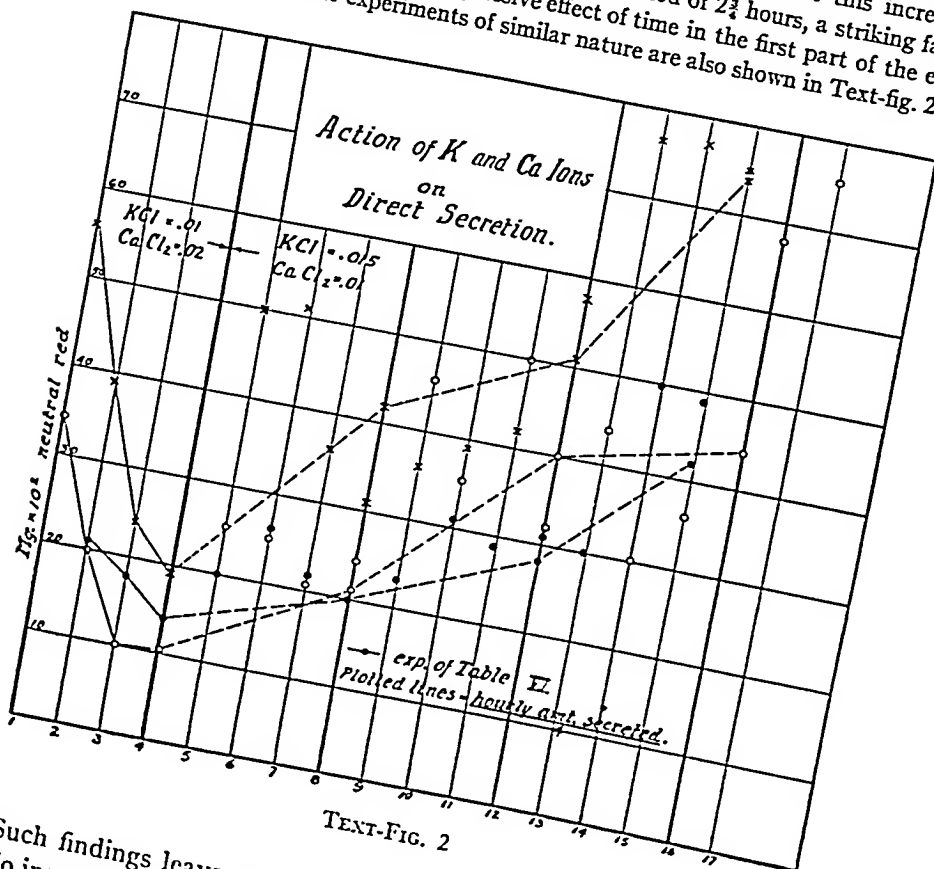
pretation is correct is supported by the fact that in the combined extravital staining experiments after the perfusion of the kidneys with Janus green the direct secretion of neutral red was prevented, yet the indirect secretory process, both in its concentrating and eliminating phases, remained intact.

As has been stated previously, under the usual conditions that follow the administration of neutral red to an animal, the two methods, direct and indirect, are concomitant and so blended in their results as to give the appearance of a single process of elimination. There are, however, variations in the relative part played by either process, and one factor which determines this is the degree of concentration of the dye in the blood vessels. With low concentrations in the blood stream the indirect method predominates; with high concentrations the indirect, though present, is less marked while direct secretion is correspondingly active.

These facts concerning the secretory activity of the renal cells have been obtained by the study of the elimination of a substance foreign to the animal's economy. The question arises as to what physiological substance may be handled by the kidney in a similar manner. It is certain that some must be so handled, for the morphological evidence of the occurrence of the indirect secretory process can be seen in the tubule cells of animals living under native conditions, and the existence of the elaborate mechanisms of direct secretion can hardly be reconciled with the assumption that, existing, they are not used. It has been suggested that certain substances, such as phosphates and uric acid, may be eliminated in part at least by the tubules rather than by glomerular filtration and an examination of this possibility by the extravital method might seem a promising procedure. But the method is not at present applicable to the problem, for as we have used it the exact conditions that obtain in the animals' blood stream are not reproduced. Such conditions are necessary to determine if a substance, bound perhaps in the blood of the living animal, may or may not filter through the glomerulus. We have called attention to this point in a previous publication (12).

Although the evidence here presented need not be discussed in relation to the direct question as to what substances are eliminated in a similar manner by the living animal, certain indirect conclusions

tion. It will be seen in Text-fig. 2 that there occurred a gradual progressive increase in the elimination of the dye which ultimately more than doubled the original output of the kidney. It is, moreover, noteworthy that this increase was a long continued phenomena, lasting over a period of 2½ hours, a striking fact when one considers the marked depressive effect of time in the first part of the experiment. Two other experiments of similar nature are also shown in Text-fig. 2.



TEXT-FIG. 2

Such findings leave no doubt that an increase in the KCl/CaCl₂ ratio increases the rate of elimination of neutral red, but this conclusion strictly examined can state no more than that an increased amount of dye entered the tubule lumen under such perfusion conditions. There are at least three ways in which such a result might come about: the inner (lumen) membrane might have become more permeable and thus allowed dye within the cell to enter the lumen more readily; the

tions between the normal secretion and pathological changes in the cells are at present being examined by the extravital method.

CONCLUSIONS

1. The elimination of neutral red by the renal epithelium is a composite process, consisting of a direct and an indirect secretion.

2. The mechanism controlling direct secretion is concerned with the permeability of the two cell membranes. These two membranes may be affected independently in the direction of either an increased or decreased permeability, with a corresponding increase or decrease in the elimination of the dye.

3. The mechanism controlling indirect secretion is concerned with the mitochondrial apparatus of the cell. By means of change in the form and constituent substance of its structures, the dye is concentrated within the cell and slowly eliminated.

4. Direct secretion, depending on the condition of sensitive membranes, is easily disturbed. Such disturbances account for the wide variations in the elimination of dye observed in the functioning of abnormal kidneys. Indirect secretion, depending on the simpler factor of the solubility of the dye in the protoplasmic constituents, continues even when the cells are severely damaged.

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neutral red in 1.25 mg. per 100 cc. After four periods the elimination of dye was 1.3 mg. per hour and the kidneys were stained a deep mahogany-red from the dye concentrated within them. At this point the bottle supplying the tubule was changed for one of dye-free Locke's solution containing a high KCl concentration of 0.015 per cent and a low CaCl_2 concentration of 0.01 per cent. The perfusion was continued for five periods and the output of neutral red determined. It is seen that a marked drop in elimination was noted in the first period following the change to clear Locke's, as great a one, in fact, as was observed in the previous experiments where the dye-free Locke's solution contained the usual amounts of KCl and CaCl_2 . Several such experiments are shown in comparison with these earlier ones in Text-fig. 1.

The conclusion is definite from these findings that no more dye is liberated from the cells by the Locke's solution containing a high ratio of KCl/ CaCl_2 than is freed by that containing the usual ratio. And this fact definitely precludes the possibility that the increased elimination noted in our previous experiments with high ratio Locke's solution could have been the result either of a liberation of the dye in the vacuolar apparatus of the cell or of an increased permeability of the inner (lumen) membrane. If the latter had occurred even without the former there must have been at least a temporary increase in the dye output in the first period following the change to the dye-free perfusion fluid, for the cells contained, as a result of their previous perfusion with dye solution, a certain amount of this material available for secretion. It was therefore only the direct secretory activity of the cell that was affected by the high ratio of KCl to CaCl_2 and the exact point of the effect of the ions must have been on the external (vascular) membrane. The effect was to increase its permeability.

These experiments allow us to draw an even sharper differentiation between the indirect secretion of neutral red with its controlling mechanism, the granulovacuolar apparatus, and the direct method of secretion, by their demonstration that this latter process has an entirely different mechanism from the former. This mechanism depends on membranes, and so delicate is its balance that it has been possible by means of special methods to not only dissociate its action from that which controls indirect secretion but to recognize disturbances in either one of the two membranes, inner and outer, that are concerned in the process of passage of dye through the cell. It is easy to understand, in view of the relative complexity of these

It is plain from these experiments that even in dead kidney cells there still occurs a concentration of neutral red above that of the perfusion fluid and that the process of concentration therefore does not depend on any vital activity of the cells but on the nature of the constituents of their protoplasm. It is possible in fact to reproduce to a certain extent all the phenomena of the indirect secretory process with a physical model.

TABLE IX
Effect of Corrosive Sublimate on Concentration of Dye in Kidney

Arterial flow	Venous flow	Urine volume	Dye	Salt in perfusion fluid	Sugar
cc. per hr.	cc. per hr.	cc. per hr.	mg. per hr.	per cent	
600	Perfusion of	tubules with	clear Locke's	solution	0
600	800	5.6	—	40	0
600	800	6.0	—	45	0
600	600	20 cc. 1/1000	sublimate to tubules	—	—
600	640	7.2	—	70	+
640	800	3.0	100 cc. Locke's solution to tubules	—	—
640	640	2.4	—	—	—
700	800	1.4	0.015	71	+
640	800	1.2	0.008	71	+
600	800	1.2	—	—	—
	640	1.2	0.007	75	+

Concentration of dye in kidneys = 22.6 mg. per 100 gm. = 18 times concentration in perfusion fluid.

The Resemblance between a Physical Model and the Indirect Secretory Processes

The model used in this demonstration is that devised by Irwin (6) and Osterhout (7) for the demonstration of the manner of entrance of dyes into living cells.

Text-fig. 3 shows the arrangement of the solutions which consisted of Locke's solution containing neutral red in the concentration used in the perfusion fluid at a pH of 7.0 as the outer fluid, chloroform, which represents the concentrating granulo-vacuoles of the cell, and the inner fluid, representing the urine in the tubules, which consisted of plain Locke's solution at a pH of 6.0. All three phases of the model were repeatedly stirred and the fluid in the inner chamber kept at a low pH by

former has to do with their application to problems of cytological method, while the latter is concerned with their significance in an understanding of the secretory activity of the renal cells.

In their general aspect the experiments with neutral red would seem to confirm the validity of the extravital method as a useful adjunct to the appearance of perfused organs, but never has the full means of the morphologist been applied with the directing idea that the results of such visual examination might be equally as valuable a part of the experiment as the findings of the physiologist. The essential matter is that neither aspect, functional or structural, shall be subordinated to the other in a correlation of simultaneous phenomena that is planned to give a complete view of the organ's total activity.

Our comparison of the manner of indirect secretion of neutral red in the extravital experiments with that observed in the living intact animal has shown a complete identity of processes. Not only do the conditions of the extravital experiment produce no artifacts in the cells, tissues or organs under examination by it, but under these conditions vital processes may proceed in exactly the same manner and by the same mechanisms, both structural and functional, as are observed in normal life. And this conclusion, taken in conjunction with our previous demonstration that the reactions of the tissues to noxious insults under the conditions of the extravital experiment are identical, both in structural change and functional response, to those observed in living animals (5, 2), would seem to establish the value of the extravital procedure among the methods of the new cytology (9, 10).

In the specific problem of renal secretion the extravital method has made possible by means of its controlled conditions an analysis of the manner of elimination of neutral red by the renal tubule cells. Secretion, which has hitherto been regarded as a unified single process, was found to be composed of two separate methods of elimination, each with its own peculiar mechanism. That under most conditions the two processes run concomitantly explains the earlier failures to recognize by the usual methods of examination the elimination as a composite process.

One of the methods of secretion is a prompt and efficient elimination

be obtained by leaving the air-dried film in Wright's stain overnight, removing and allowing stain to evaporate as described, then washing rapidly with buffer and drying with the electric fan.

FINDINGS

By this technic a sharp color differentiation is obtained. If duration of staining and of washing are correctly timed the bacterial somata stain blue, the capsular substance pale, purplish pink and the periphery of the capsule (capsular membrane?) deep purplish pink (Fig. 1). The behavior of the capsules toward the stain is not, however, absolutely constant and the following variations in result occur. Occasionally a number of types of staining may be found on one slide. But, as a rule, one or two types predominate, and often only one type is seen.

1. When the specimen is overstained, the entire organism (soma, capsular substance and capsular membrane) may be dark purple, the bacterial body being thus obscured if not entirely hidden and the capsule, as such, not distinguishable (Figs. 2 and 9).

2. The capsular membrane may be deep pink and the soma blue. Lying between them the colorless or nearly colorless capsular substance then appears as a clear space (Figs. 3-5). This is the way certain species (*e.g. Clostridium tetani*) almost always behave; only occasionally is their capsular substance definitely stained.

3. When the bacteria being studied occur in chains or segments the capsular partitions which separate the individual components may be stained all the way across the organism (Figs. 11, 17, 26, 34), or part way across (Figs. 1, 2, 7).

4. Granular precipitation of stain may occur on the capsular membrane. The pink line of the membrane is thus either entirely obscured by purplish granules or it is to be seen, at the periphery of the capsular space, with granules lying upon it (Figs. 6, 7).² Sometimes the appearance suggests that the capsular membrane is, in places, missing; but careful examination of these apparent gaps between granules will

² The photomicrograph represented in Fig. 7 was deliberately made from one of the poorest fields in the specimen in order to show individual bacteria with granular deposit on the bacterial membranes. In the specimens of this organism as usually stained and indeed in other fields of this particular slide capsular staining was excellent and the pneumococci looked like those shown in Fig. 1.

with neutral red. This property is not possessed by the original mitochondria, and by it the dye is concentrated within the cell body during indirect secretion. In this sense the vacuoles function as Gurwitz' *condensoren* (11). If a replacement of filaments by vacuoles has already occurred in the renal cells as a previous response to some other stimulus, then these preexisting vacuoles function as condensers of the dye.

The indirect secretory process is not easily affected in its slow and constant elimination. No factors were found that definitely increased it in our experiments, and on the other hand it was observed to continue undisturbed when direct secretion had been almost completely eliminated by the action of toxic substances. It was found indeed that dead cells, killed by corrosive sublimate, still were able to concentrate within themselves a certain amount of the dye above that in the perfusion fluid, and the urine from such dead tubules, though identical to the perfusion fluid in other regards, contained some dye, in spite of the fact that little or no dye could have been present in the glomerular filtrate under the conditions of the experiment. The essential processes involved in indirect secretion are therefore not vital in any sense of the word, but depend on the chemical or physical constitution of the tubule wall that remains essentially unaltered even after its cells have been killed by the mercuric salt. That the dead cells are less efficient than the living may well be due to the fact that they are less permeable to the dye after coagulation of their protoplasm, as well as to the fact that after this severe damage the condensing substances are no longer arranged in the previously efficient emulsion-like pattern of the granulovacuolar droplets which present a very large effective surface. Further evidence that indirect secretion depends on simple physical or chemical processes is suggested by the fact that models which duplicate its action may be easily constructed.

Another illustration of the relation between the instability of direct and stability of indirect secretion is particularly well shown by the results of extravital staining with Janus green. It will be remembered that this dye enters the cell and is stored in the granulovacuolar apparatus but that it does not appear in the urine. Apparently its toxicity is sufficient to prevent direct secretion but inadequate to stop the concentration processes of the indirect secretion. That this inter-

Facts of these kinds have taught us to be extremely chary about concluding that because capsules have not been stained they are not present, any more than one would jump to the same kind of conclusion concerning the flagella.

Sometimes the failure of the capsule to stain is easily explained. For example, if washing with buffer solution be done before evaporation of stain has gone far enough, all color may be removed from the capsules and they will be invisible. The same thing may happen if the stained films are washed too long. However, for a number of other striking irregularities encountered no completely satisfactory explanation is at hand. It seems probable that uneven distribution of material taken up from the cultural media, or differences in the character of these materials, may be among the factors concerned. Is it not at least possible, too, that when capsules are supposed to be "developed" by residence in a particular environment they are actually only swollen or even only impregnated with materials in the absence of which they are incapable of taking or retaining stains?

A number of the different ways in which capsules behave toward the stain used in our technic are shown in Fig. 2 which represents a smear of *Klebsiella pneumoniae* Friedländer. It will be noticed that in some instances soma, capsular substance and capsular membrane are all stained deeply and alike; in these individuals, the structural arrangement is not to be made out. In other cases, soma, capsular membrane and the nearly colorless, capsular substance are all clearly seen. Between these two extremes, a number of variations in depth of staining will be noted. These obviously cannot be explained as technical in origin: they must arise from differences among the individual organisms themselves. If they are thus encountered in one preparation, it is not strange that variations of a similar kind are met with in the examination of different preparations.

Capsular Membrane.—With our technic, as indeed with other methods of staining the capsule, the stain—except in the case of greatly overstained organisms—is apt to be more concentrated at the periphery of the capsule than elsewhere.

In some, but not in all, species it is easy, if one wishes, to stain the entire capsular substance (e.g. the overstained organisms in Fig. 2; also in Fig. 9). But the specimens are more satisfactory for study

may be helpful for further work. For example, recognition must now be taken of the fact that secretion by the renal cell is a composite as well as complex problem. As we have shown, one method of secretion may exist without the other and the complications that might arise in the interpretation of results unless this fact is recognized are evident.

It is the indirect method of secretion that interests us at present. It is a mechanism ideally fitted to effectively remove from the blood stream a non-filtrable substance which is present intermittently and in relatively low concentration. This is done by a process of storage within the kidney cells followed by a slow but ultimate elimination. Some of the normal deleterious end-products of the animal's metabolism may be included in such a category, but evidence seems to accumulate that they are eliminated more directly by glomerular filtration.

There is another category of deleterious substances, however, which must be eliminated and this includes both the end-products of disturbances in the organism's metabolism, that is of disease processes, and those toxic substances which fortuitously enter as casual contaminants of the food supply. We know that the kidney concentrates within itself and eliminates into the urine such grossly toxic exogenous substances as the heavy metals. The analogy with the indirect secretion of the relatively non-toxic neutral red is striking.

By such a concept indirect secretion as we have described it would become a protective mechanism in a stricter sense than can be applied to elimination of toxic metabolites. It might indeed be considered a pathological process, though the frequency with which all living organisms must call upon some method to free themselves from the results of such every day disturbances as we have mentioned, would make its occurrence almost a normal phenomenon. And it is of particular interest in this regard that pathologists have long sought to connect a certain frequently occurring pathological process, cloudy swelling, with the hyperactivity of cells, and that in the kidney the hyperactivity has been assumed to be a secretory one. Furthermore, the morphological evidence of cloudy swelling is a disturbance in the mitochondrial and granular apparatus of the cell and a typical part of this disturbance is the appearance of Gram-positive granules (13, 14). Such granules we found to be a constant evidence of a secretory activity in the renal cells unassociated with any toxic phenomena. These rela-

flagella have been unsuccessful. The matter is further discussed under Critique. While the majority of specimens (as the illustrations show) are clear enough, we are well aware that the evidence presented, being purely tinctorial, is subject to the hazards of tinctorial methods. We have, for purposes of accurate discussion, avoided evasions and used the word "capsule" to describe the appearance our method brings out in the belief that this structure is probably though not certainly a capsule. But we are fully aware that it ought not to be definitely regarded as a capsule until the evidence is fortified, if possible, by evidence not tinctorial in character.

Modifications of the Technic.—The capsule may also be demonstrated by the use of certain modifications of Wright's stain.

For example, the substitution of erythrosin for eosin in the Wright formula has in a number of instances proved satisfactory, although specimens stained with this modification are not permanent since the erythrosin tends to fade. Excellent results have also been obtained with Wright's stain made up with ethyl instead of methyl alcohol, and with Wright's stain in which glycerin was used as the solvent.

In addition to such modifications as these we have, in the study of certain organisms, used with some success MacNeal's tetrachrome stain (Fig. 13), Giemsa's stain (Fig. 14)³ and Casares Gil's method for flagella (Fig. 15). Of the last, more will be said later in discussing the relations of flagella to capsule (see page 500).

Capsulated Organisms Studied.—The merit of our technic was established by the study of 3 of the best known "capsulated" organisms: *D. pneumoniae* (S strains of Types I, II and III), *B. anthracis* Koch (from the mouse's spleen) and *Klebsiella pneumoniae* Friedländer.

Of *D. pneumoniae*, 28 strains from 5 different sources (Avery, Rockefeller Institute; Gay and Dawson, Columbia; Park, New York City Bureau of Laboratories; DuBois, Bellevue) were used. All were well established, virulent, definitely capsulated strains. A number of representatives were used which were known to secrete the specific soluble polysaccharide. The capsule, as stained by our method, was identical in site and size with the capsule as stained by the ordinary methods (Fig. 7). When films were made from the peritoneal exudate of mice which had received intraperitoneal injection of Type III S the capsules appeared swollen, exactly as they do when stained by the usual capsular methods (Fig. 16).

³ Used also by Foth and others. See Foth, E., in Kolle, W., and von Wassermann, A., *Handbuch der pathogenen Mikroorganismen*, Jena, Gustav Fischer, (Kolle, W., Kraus, R., and Uhlenhuth, P.), 1929, 9, Liefg. 34, 782.

EXPLANATION OF PLATE 32

1. Segment II of the kidney from the experiment of page 474 whose renal pelvis was killed extravitally by corrosive sublimate. Necrosis and desquamation are seen with pycnosis and absence of nuclear staining in the dead tissue. Neutral red was nevertheless concentrated in these dead tissues. Magnification $\times 525$.

technic (such as Boni had used) which introduced a number of sources of possible error from artifacts (*e.g.* Hamm⁶). Still we find Carpano⁷ in 1913 making the statement that the "capsule is nothing other than a constant structural peculiarity which it is not always possible to recognize because of imperfect technic."

Ideas like Carpano's were not, however, generally held. It was still usually thought that the division of bacteria into "capsulated" and "non-capsulated" groups represented the facts as they exist in nature; and even in the case of the virulent organisms, capsules were regarded by many (as Babes had suggested) not as essential structural units but as a defense mechanism (Sauerbeck's "bacterial immunity through structural adaptation"⁸). "Highly virulent bacteria," wrote Eisenberg, "surround themselves in the infected host with a thick capsule which is lost when the bacteria are transferred to artificial media."⁹

This general conception (which fails perhaps to take sufficiently into account the fact that saprophytic bacteria may also surround themselves with "the deadly capsule") may be said to be the one which still obtains. Yet data have been for some time accumulating which have thrown considerable doubt on it. Many scattered reports have appeared in which the observation of capsules, where they had not previously been seen, is recorded. Thus, Huntoon describing a new technic for staining capsules wrote: "This method has been tried on streptococci, staphylococci, members of the Gram-negative group and many flagellated organisms and has in no instance shown a capsule on these organisms but a similar structure may be demonstrated on all by means of a special technic which will be the subject of a subsequent report."¹⁰

No further report ever appeared, but we have seen photomicrographs of Huntoon's specimens and there is little doubt that he did stain capsules on a number of "non-capsulated" organisms.

In 1925 Cooper¹¹ showed definitely, as Marrassini¹² had done in 1913, that *Eberthella typhi*, *Escherichia coli*, *Salmonella enteritidis* and a number of other organisms are, at least under certain conditions, capsulated.

In our own study of the "non-capsulated" group, organisms (like *D. pneumoniae* R) were included which are universally regarded—and on apparently convincing evidence—to be devoid of capsules. Others (like *Escherichia coli*) were included of which—although the species is usually regarded as non-capsulated—strains or mutants

⁶ Hamm, A., *Centr. Bact., 1. Abt., Orig.*, 1907, 43, 287.

⁷ Carpano, M., *Centr. Bact., 1. Abt., Orig.*, 1913, 70, 42.

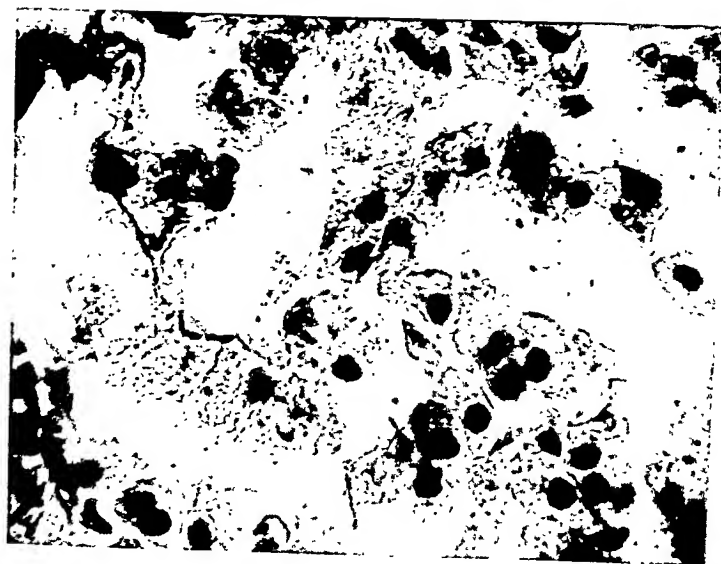
⁸ Sauerbeck, E., *Z. Hyg. u. Infektionskrankh.*, 1909, 63, 313.

⁹ Eisenberg, P., *Centr. Bact., 1. Abt., Orig.*, 1908, 47, 415.

¹⁰ Huntoon, F. M., *J. Bact.*, 1917, 2, 241.

¹¹ Cooper, M., *J. Infect. Dis.*, 1925, 36, 439.

¹² Marrassini, A., *Centr. Bact., 1. Abt., Orig.*, 1913, 71, 113.



Proteus vulgaris Hauser (Fig. 25). 1 strain from Torrey.

Alcaligenes faecalis Castellani and Chambers (Fig. 6). 1 strain from Torrey

Streptococcus faecalis Andrewes and Horder (Fig. 26). 6 strains from Torrey.

About all these organisms structures were demonstrable not only by our technic but in a number of instances also by the method of Giemsa, MacNeal and Casares Gil which had the appearance of the structures usually spoken of as capsules.

We have also studied the following cocci.

Streptococcus haemolyticus.

Streptococcus viridans.

Neisseria gonorrhoeae (Neisser) Trevisan.

Neisseria intracellularis (Weichselbaum) Holland (meningococcus).

Staphylococcus aureus Rosenbach.

This group we have not, up to the present time, subjected to the intensive scrutiny—either as regards number of strains examined or number of films made from the strains studied—which has been devoted to the other organisms upon which we are reporting. Definite conclusions are not therefore warranted concerning all the members of the group. They will be reported upon later.

The study has also included examination of the following members of the acid-fast group.¹⁶

Mycobacterium tuberculosis (hominis) Koch.

Mycobacterium tuberculosis (bovis) Th. Smith.

Mycobacterium tuberculosis (avium) Straus and Gamelsia.

Mycobacterium smegmatis.

studied both the S strain, and the R strain dissociated by Tyler. Capsules were demonstrated on both with equal ease. It is interesting to learn from Olitsky (personal communication) that the presence of capsules on the S form had been strongly suspected by Tilden, in his laboratory; and that Tyler had later suspected their presence on the R form. The observations were not considered sufficiently well established to justify report and have never been published. Olitsky informs me that our findings, as regards the presence of capsules on both S and R forms, as well as regards the relation of capsule to flagella, have recently been confirmed in his laboratory.

¹⁶ All but *Mycobacterium tuberculosis bovis*, which came from the New York City Bureau of Laboratories, were furnished by Dr. M. C. Kahn of the Cornell Medical College.

A STUDY OF THE BACTERIAL CAPSULE BY NEW METHODS*†

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PLATES 33 TO 35

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The purposes of this communication are to present a new method of staining bacterial capsules, to record the demonstration by stains of their presence on a number of supposedly non-capsulated organisms, to point out the structural relation of flagella to capsules and to consider the bearing of these observations on present day conceptions of the nature of the capsule.

Technic

The best results have been obtained when young organisms (not more than 18 hours old) from a culture on solid media were used for study, although capsules have also been stained in smears from broth cultures and (in the case of *Diplococcus pneumoniae*) from sputum. Use is made only of stain and fixative, which are applied together. No steps are taken (like preliminary treatment with serum, acetic acid, nutrose, etc.) which are designed to render the capsule more readily demonstrable but are likely also to produce somewhat more artificial conditions. Films are made from aqueous suspensions of the bacteria and these are air-dried. They are then covered with 10 drops of freshly filtered Wright's stain which is left on until it has evaporated nearly, but not quite, to dryness, when a purplish pink replaces the original color of the Wright's stain. It is probable that staining of the capsule occurs just at this instant. The evaporation usually requires about 3 minutes. The stain is washed off as rapidly as possible with Clark and Lubs' buffer pH 6.4 to 6.5 and immediately dried with a fan without blotting.¹ Sometimes the capsule is more satisfactorily stained if the film is rapidly washed with distilled water after the buffer has been used. Excellent specimens may also

* Work aided by a grant from the Josiah Macy, Jr., Foundation.

† Preliminary reports of this work have appeared in: *Proc. Sec. Exp. Biol. and Med.*, 1932, 29, 514, 515, 996.

¹ In this laboratory bacterial films are practically never blotted. We have found a hot air electric fan of the kind employed for drying hair very useful.

may be only a light effect. Quite similar appearances are often to be noted about any black lines in high power photomicrographs (*e.g.*, the flagella in Figs. 21 and 35; the halos around and quite *outside the capsules* in Fig. 35, and a similar halo seen in Fig. 23 *a* which is the photomicrograph—2000 magnification—of one of the scratched lines on a Leitz ruled slide). Appearances of this sort should make one accept with caution Koch's dogma to the effect that only photomicrographs are of scientific value for the representation of bacteria.¹⁷ That photomicrography at high powers—whether of stained or living cells—is not a method of impeccable exactitude, but contains pitfalls like any other technical maneuver, is perhaps not sufficiently appreciated. Note in this connection Fig. 14. No one would suspect that the specimen from which this photograph was made showed capsules beautifully stained; the camera simply failed to record adequately the contrasts of the differential stain (red and blue) which produced in the eye a clear-cut image of a capsule with sharply defined periphery.

While, for descriptive purposes, the term "soma" is at present a useful one, it would be unfortunate if its employment implied that the structure it describes is a more essential, constant or important part of bacteria than other parts. Indeed, observations like those here reported suggest the possibility that some relation may exist between soma and capsule like the relation between nucleus and cytoplasm. This hypothesis we are investigating. While it may turn out to be well founded we cannot at present offer any scientific evidence whatever for its correctness.

It is worth while to call attention to the well known but somewhat neglected fact that the somata of any given organism may, when stained, vary a good deal in apparent size (particularly in caliber) not only among different films but also among different individuals seen in a given film. Careful scrutiny of practically any stained specimen will bear this statement out, at least for the majority of bacteria. These variations are clearly to be seen in the specimens shown in Figs. 1, 2, 4 and 26. The point is brought up here because observers are sometimes perplexed, as to the interpretation to be put upon what appear to be capsules, if they chance to find somata (in a specimen stained for capsules) smaller than "normal." What usually happens in such cases is that, when apparent capsules are found where expected, no attention whatever is paid to size of soma; but when found on bacteria supposed *not* to possess capsules, size of soma is carefully

¹⁷ Koch, R., *Mitt. k. Gsndhtsamte*, 1881, 1, 10.

almost always show the fine pink line of the membrane on which the granules probably rest (Fig. 22).

5. Occasionally the so called staining is "negative." The somata are stained blue but capsular substance and capsular membrane are quite unstained. The capsule therefore stands out sharply as a clear space against the somata and the purplish background (Fig. 8).

6. Occasionally no capsules at all can be seen. It sometimes happens indeed that it is impossible to demonstrate them in a film from a given suspension, while examination of a second film from the same suspension reveals them clearly. Not infrequently the specimen stains irregularly; and, when this occurs, the best stained capsules are apt to be at the edge of the film. This phenomenon has been noticed also by other observers working with other staining methods.

7. Not all the species on which we have demonstrated capsules react with equal readiness toward the stain. With some, satisfactory results are obtained almost constantly and usually with little difficulty. This is true of *Klebsiella pneumoniae* Friedländer, of *Clostridium welchii* and of *Clostridium tetani*. It is also true of many strains of *D. pneumoniae*.

In other instances, attempts to stain the capsules are successful only after repeated failures. The capsules in such cases appear to be capable of taking or retaining the stain only when every single factor (duration of exposure to stain, amount of washing, age of culture, pH of environment, chemical constitution of fluids in which the bacteria are suspended and probably other unknowns) is favorable.

The behavior of many capsules toward stains appears thus to be easily upset and often by factors which cannot be assigned. This capriciousness, as compared with the dependable staining behavior of somata, is a characteristic which they share with flagella. It is established by certain other facts in addition to those already referred to. Thus, we have examined films, stained by our technic, which showed no capsules whatever but in which, on restaining, the structures were clearly demonstrable. We have also made the observation, in the case of *B. anthracis*, that a readily demonstrable capsule could be made apparently to disappear by simply exposing the organisms to sodium chloride; the "dissolved" capsules reappeared when the salt was washed away and the film again stained and examined.

types. Between this ring and the bacterial body there is often a quite clear space; but not infrequently this space is stained pink. The appearance of the R forms is exactly the same as that of the capsulated S forms; and the "structure" seen about the R forms occupies exactly the position of the capsule of the S forms.¹⁹

The same is true of the other organisms supposed not to be capsulated of which we have spoken. The stained pink line surrounding them, with the clear (sometimes light pink) space between it and the soma corresponds to the typical picture of a capsule.

Clarity in this field has given place to confusion because bacteriologists have failed to establish, adopt and then adhere to any standard. Three factors appear to have contributed to the confusion:—

1. It is well known that in the case of some bacteria which produce a sticky growth (*Klebsiella pneumoniae* Friedländer, *D. pneumoniae* III S and others) a secondary zone is sometimes observed quite outside the capsule. This zone is not constant, is usually irregular in shape and has no sharply marked periphery, though the stain may be denser at the edge than elsewhere. It has none of the appearances of an organized structure but suggests on the contrary a layer of amorphous material adherent to the outer surface of the capsule (Figs. 1, 9, 30). Not infrequently we have observed a similar picture in studying flagellated bacteria and there is reason for believing that in some of these instances the outer zone represents stain adherent to flagella, sometimes perhaps to the stumps of broken flagella (Figs. 10 a, 11, lower organism).

2. Confusion has become particularly frequent since the introduction by Zettnow²⁰ of terms, new to this field, and their adoption by Toenniesen²¹ in his important chemical studies of the capsule of *B. friedlaenderi*. Zettnow had introduced into bacteriology and defined the perhaps not altogether fortunate terms "endoplasm" and "ectoplasm." The former, he had stated, stains blue by the Romanowsky method and readily by the usual aniline dyes. The latter is not stained at all by Romanowsky or ordinary dyes; it is stained, if at all, only with difficulty and after mordanting. Endoplasm is that part of the organism usually spoken

¹⁹ The staining technic described in this paper was put to a test as to availability for routine use, through the kindness of Dr. Stuart Mudd. The students in his class in bacteriology at the University of Pennsylvania were given R strains of pneumococcus, from his laboratory, to be stained by our method. In a number of the more successful slides, one of which we have seen, capsules were very neatly demonstrated (personal communication).

²⁰ Zettnow, E., *Z. Hyg. u. Infektionskrankh.*, 1897, 24, 72; 1899, 30, 1.

²¹ Toenniesen, E., *Centr. Bakt., I. Abt., Orig.*, 1912, 65, 23; 1913, 69, 391; 1914, 73, 241; 1921, 85, 225.

and more suitable for photography if the stain is (save at the periphery or capsular membrane) washed out of the capsular substance, either completely (Figs. 3-5, and 11), or almost completely (Figs. 1, 10 *a*). The sharply stained pink line which is then seen surrounding the blue-stained soma, from which it is separated by a clear unstained space (the capsular substance) we have referred to as "capsular membrane."

The term "capsular membrane" is useful for descriptive purposes. It is possible, although not proven, that the presence of a membrane does actually account for what we see. Yet it is not altogether clear just how this picture is produced. The capsule must surround the soma like a sac. How does it come about then that, in many organisms, the linear periphery only, of this sac as it lies flattened out on the slide, is deeply stained, while no dye whatever is seen in the rest of it? Perhaps its attachment to the slide at its edge leads at this site to retention of dye which is more easily washed out of the main portion of the capsule; for it is well known that some stains tend, as they evaporate, to accumulate at any edges encountered. Witness, for example, the frequently observed phenomenon (already referred to) of good capsular staining at the periphery of a film in the rest of which no capsules at all are to be found.

We can, however, suggest no entirely satisfactory explanation for the linear staining of the capsular membrane. Though a matter of common observation, it appears to have been accepted by bacteriologists without inquiry.

Criticism of the Technic.—In a large proportion of specimens examined by this technic good staining occurs, and structures about the somata are seen, differentially stained, which no one could, we think, describe as anything but capsules if that term is to be used at all. On the other hand the picture is sometimes much less satisfactory. Precipitation may occur, in granular and sometimes in annular form, and in these slides one is not infrequently considerably perplexed in considering many individuals to say whether what is seen about the somata should be interpreted as valid capsule or as some kind of artifact. The question of artifact has, of course, been kept constantly in mind. Attempts made to produce the capsule-like structure, on non-bacterial particles (collodion particles, red and white blood cells) as well as on organisms displaced from their apparent capsules and on

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²¹ Toenniessen, E., *Centr. Bakt., 1. Abt., Orig.*, 1912, 65, 23; 1913, 69, 391; 1914, 73, 241; 1921, 85, 225.

Of *B. anthracis* Koch, 6 strains were studied. These had been obtained either from the New York City Bureau of Laboratories (Dr. Park) or from the American Type Culture Collection. The authenticity of all had been established during years of experimentation in this laboratory. All were highly virulent for mice. All showed, when examined from the mouse's spleen (either by our technic or by the usual methods) readily demonstrable capsules (Fig. 3).

Of *Klebsiella pneumoniae* Trevisan (*B. friedlaenderi*) observations were made on 8 strains: 6 from the New York City Bureau of Laboratories and 2 from Bellevue Hospital (service of Dr. DuBois). All had been isolated from the human being and were typical in every respect. The capsules, as stained by our method, corresponded in every way with those seen when the usual capsular stains are used (Figs. 2, 9).

Presence of Capsules on "Non-Capsulated" Organisms.—The study was next extended to include the "non-capsulated" group, a group which—though now known to be less sharply demarcated than was formerly thought to be the case—is still a fairly generally recognized bacterial class.

For some years after Friedländer, for the first time, demonstrated the presence of capsules on pathogenic organisms it was thought that this feature was peculiar to the bacterium which came to bear his name, and to a few others (like *B. anthracis*) which were found to resemble it in this structural respect. Since capsules could not be demonstrated on the great majority of remaining organisms, one of the firmest teachings of bacteriology thus came to be that bacteria could be sharply divided into the "capsulated" and the "non-capsulated" groups. Although this idea before long began to undergo modification, it is in the main still widely accepted. Many believe that, in the case of certain bacteria like *B. friedlaenderi*, the capsule is an essential part of the organism, practically always present; while in another much larger group, capsules are never to be seen or only under quite special conditions.

There were early premonitory guesses, based in some instances on sound observation, that the case was not quite so simple as this. Migula,⁴ for example, in 1896, at a time when only a few organisms had been shown to possess capsules, prophesied that more and more of them would be put into the capsulated group as the field was more carefully examined; and that probably all were in fact capsulated, though the capsules would doubtless be found to vary greatly in size.

The pendulum occasionally swung all the way in the direction thus indicated by Migula; and some observers (for example Boni⁵) went so far as to state that they had demonstrated capsules "on all bacteria taken from any media." Most bacteriologists considered it precarious to draw conclusions so sweeping from a

⁴ Migula, W., *Deutsch. tierärztl. Week.*, 1896, 4, 28.

⁵ Boni, I., *Centr. Bact.*, I. Abt., 1900, 29, 705.

often it is simply stated "capsules are of two kinds, the true capsules produced by the organism as a kind of secretion, and culture capsules forming in the culture-media and deposited on the outside of the organism."²²

3. A third source of confusion comes from the notion which has arisen that the capsule is somehow to be thought of as a structure only of a certain size, and that anything smaller than this must be something else. It goes without saying that the standard must of necessity be quite arbitrarily chosen.

However, capsular dimension is perfectly well known to be a variable. Swelling (e.g. of the capsule of *Pneumococcus* III S after residence in the peritoneal cavity of the mouse) is a well established phenomenon; the capsular membrane in such instances lies further from the soma than "normally." Exposure to nutrose also appears to cause similar enlargement, as does exposure to glacial acetic acid which, in the Welch technic, is added for this very purpose.

Capsules which are thus able to swell must also be capable of shrinking. The distance between bacterial body and capsular membrane will then be diminished; capsular substance will be scant or wanting, and the membrane may lie practically upon the soma. It is well known that shrinkage of this sort occurs when bacterial films are heated, or fixed with sublimate; its occurrence under such conditions is, according to some observers, more frequently seen in bacteria taken from culture than in those taken from the animal body. The apparent size of the capsule is also known to vary, in India ink specimens depending on whether these are examined in water or oil. There appear to be thus a number of reasons for thinking that the capsule may in fact be a sac, now collapsed, now swollen. At any rate it is certainly illogical to make size a criterion as to whether a structure known to vary in size shall be designated as "capsule."

The facts of the bacterial structure of *D. pneumoniae* as brought out in this study are schematically represented in Text-fig. 1. The specimens, on which these diagrams were based were stained by our technic, in three colors: blue, pink and several shades of purple. These are represented by black and gray.

Relation of Capsule to Flagella

There is still considerable diversity of opinion concerning the structural attachment of the flagella, and some authors (Fuhrmann²³ and Yamamoto²⁴) even yet regard them as directly connected with the bacterial body. This in spite of their well established chemical differences from it (as shown for example by the van Ermengen technic) and the differences in their antigenic behavior. Additional

²² Ford, W. W., Text-book of bacteriology, Philadelphia and London, W. B. Saunders Co., 1927, 39.

²³ Fuhrmann, F., *Centr. Bakt.*, 2. *Abl.*, 1910, 25, 129.

²⁴ Yamamoto, J., *Centr. Bakt.*, 1. *Abl.*, *Orig.*, 1910, 53, 38.

which possess capsules have repeatedly been shown to exist (e.g., among others by Smith¹³ and Cooper¹¹). Others (like *B. anthracis* Koch) were included which, while known to "develop" capsules in the animal body, are supposed to be non-capsulated in culture; as well as some species (like *B. subtilis* (Ehrenberg) Cohn) on which capsules have been seen only rarely and then as a rule in specimens taken from some quite special environment (e.g., bread dough).

The study comprised the following organisms. All either had been recently isolated and identified by competent bacteriologists or were checked up by us in accordance with the specifications of the Descriptive Chart Prepared by the Committee on Bacteriological Technic of the Society of American Bacteriologists. With the exception of the minor variations noted all were true to type:—

D. pneumoniae R strains Types I, II and III (Figs. 1, 17 and 18). 18 strains from Avery, Gay, Dawson, Plummer and Mudd.

B. anthracis Koch (from agar culture) (Fig. 4). 6 strains from New York Bureau of Laboratories and A. T. C. C.

Serratia marcescens Bizio; *B. prodigiosus* (Fig. 19). 4 strains from A. T. C. C. *Pseudomonas aeruginosa* (Schroeter) Migula; *B. pyocyaneus* (Fig. 20). 2 strains from Elser and New York Bureau of Laboratories. Both typical except that no pyocyanin could be extracted.

B. subtilis (Ehrenberg) Cohn (Fig. 11). 2 strains recovered in this laboratory from hay infusion.

B. subtilis var. *viscosus* Chester (Fig. 21). This variety is described by Ford,¹⁴ but is not described by Bergey. 1 strain recovered in this laboratory from hay infusion.

Erysipelothrix rhusiopathiae (Fig. 22). 1 strain isolated in this laboratory from a mouse dead of experimental anthrax. By most of the important tests (including serological reactions) true to type; not absolutely typical.

Eberthella typhi (Fig. 10b). 1 strain from Torrey.

Escherichia coli (Figs. 13, 23). 3 strains from Torrey.

Clostridium tetani (Nicolaier) Holland (Fig. 5). 4 strains from Steffen (Elser's laboratory).

Bacterium granulosis; S and R strains (Figs. 12, 24).¹⁵

¹³ Smith, Th., *J. Exp. Med.*, 1927, 46, 125-166.

¹⁴ Ford, W. W., Text-book of bacteriology, Philadelphia and London, W. B. Saunders Co., 1927, 664.

¹⁵ The cultures used for study were kindly furnished by Olitsky and were made from an organism originally isolated by Olitsky and Tyler from a case of human trachoma (*Science*, 1930, 71, 561). In the first description of *Bact. granulosis* by Noguchi no mention was made of a capsule (*J. Exp. Med.*, 1928, 48, suppl. 2, 16) nor has its presence been suggested in any subsequent publication. We

violet and the flagella green. The work of Weiss,²⁶ who studied the different ways in which the bacterial constituents react toward acid and basic dyes, provides further testimony in the same direction. Migula⁴ noticed many years ago that capsules not visible in ordinary stains may become visible in flagellar stains (*e.g.* on the hay bacillus) and stated "that the capsule appears to consist of the same material as the flagella which arise from it."

Our own studies, particularly of specimens stained by the method of Casares Gil, present convincing evidence that—in many instances, at least—the flagella have no connection whatever with the bacterial body but are attached only to the capsule. The testimony on this point furnished by such specimens as those represented in Figs. 15, 21, 31 is convincing enough; and it is fortified by the study of empty capsules. One of these is represented in Fig. 32. The well stained capsule is clearly seen and arising from it the well stained flagella. No bacterial body is present; it has been popped out of the capsule; what appears to be the place where it once lay is readily seen on close inspection. This phenomenon is frequently observed. It is represented again in Fig. 33 where both the bacterial body and the capsule from which it has been tipped are clearly seen. The capsular attachment of the flagella in this instance is again obvious and the apparent former site of the bacterial body is here also to be made out by careful scrutiny. Emptying of the capsule by popping out of the soma is again represented in Fig. 11. In staining this specimen our capsular method, but no flagellar stain, has been used. One bacterial body lies within its capsule as usual. The other has been tipped out.

If it be objected that the bacterial tipping represented in these instances is apparent not real, and that the bacterial bodies which seem to lie over the capsules they have left are only occupying this position by chance (having been lodged there during the process of making the film) the objection is met by the specimens shown in Fig. 34. Two chains of enterococcus stained by our method are shown; the bacterial bodies are stained blue, and the capsular membrane, pink; the capsular substance is nearly colorless. The bacterial bodies of the right two-thirds of each chain lie within the capsule. The left one-third of each chain (*i.e.* two individual cocci) has been tipped out of its capsule which in this portion of the chain is empty. This is

²⁶ Weiss, E., *J. Infect. Dis.*, 1928, 43, 228.

Mycobacterium phlei.

Mycobacterium isolated from garter snake by Aronson.

Mycobacterium isolated from iguana by Aronson.

Rat leprosy bacillus No. 368 (National Tuberculosis Association).
A special technic was employed for this group. The organisms were first definitely established as acid-fast by the Ziehl-Neelsen method. Films were then made from aqueous suspensions and these, after being air-dried, were allowed to stand overnight in carbolfuchsin. The carbolfuchsin was then washed off with water, and acid alcohol applied in the usual way. This was washed off with water, the specimen dried with a fan and then stained by our capsule method.

On 1 acid-fast strain (*Mycobacterium tuberculosis bovis*, New York City Bureau of Laboratories Cow 4) we have with this technic demonstrated structures which have the appearance of a capsule (Fig. 27).

The structures also showed up clearly when films from aqueous suspensions of this organism were stained by the following technic: dried in air; exposed to Casares Gil mordant for 3 or 4 minutes; washed with water; stained for 3 or 4 minutes in steaming carbolfuchsin; allowed to stand in this stain until cool (Fig. 28). One striking feature of these specimens is the frequency with which the organisms lie in an eccentric position in the capsule. This feature is not, however, peculiar to *Mycobacterium tuberculosis bovis*; it is occasionally encountered in the study of other organisms (e.g., *Eberthella typhi* Fig. 10 b, *B. subtilis* Fig. 11).

Concerning the other members of the acid-fast group we cannot, until the termination of investigations now under way, make any positive statement.

Definition of Capsule

The soma or bacterial body is easy to define. It is that part of an organism which (save perhaps in the case of the acid-fast group) stains readily with methylene blue, crystal violet, acid fuchsin and other similar dyes, without the aid of mordants. That is to say, it is the only part which is usually seen in the routine examination of bacteriological films. Fig. 29 shows the soma of *B. anthracis* stained with methylene blue and photographed at the same magnification as the illustration (Fig. 4) made to demonstrate the capsule.

The halos about the somata, caught by the camera in the photograph represented in Fig. 29 were quite invisible to the eye. It is tempting to interpret such appearances as caused by capsules, a deduction of dubious justification sometimes made. Such an explanation may be the correct one. On the other

body in the individual in the upper left hand corner of Fig. 36 *a* consists actually of soma plus capsule is thus evident and the capsular attachment of the flagella in this organism is thus well established.

While the relation of flagella to capsule just described and as represented in the illustrations is certainly often the relation which actually obtains, it is quite certainly also not the constant one; for we have seen many flagellated cells on which no capsule whatever was present, and in these the flagella naturally arose directly from the bacterial body. Our observations as to the capsular attachment of flagella agree with those of Babes who in 1895 published a paper on the subject which has never received the recognition it merits.²⁷ Olitsky informs us (personal communication) that his recent study of *Bact. granulosis* confirms these ideas, so far as this organism is concerned.

It is interesting to note in this connection the statement of Cooper¹¹ that "the organisms which are normally motile were found to be non-motile during the period when capsules are demonstrated." The implications of this statement are quite at variance with our findings.

Relation of Capsule to Cortex

Observations were reported in 1927 and 1929²⁸ which suggested that many Gram-positive organisms possess a Gram-negative medulla and are Gram-positive only at the surface or cortex (Fig. 37). The evidence presented at that time for a structural arrangement of this kind, if not absolutely conclusive, was highly suggestive. It has since been fortified by further testimony. We have been able—but not with constancy—to produce bizarre swollen forms of *B. anthracis* in which the medulla and cortex are exaggerated in thickness. In some of these the former, stained pink by Burke's method, can be clearly seen surrounded by the latter, stained bluish black. In segments where the Gram-positive material has been stripped away the medulla is seen passing into the cortex of the next adjacent segment like a piston into a cylinder.

The picture of Gram-negative medullae surrounded (in part or completely) by Gram-positive cortices, which we are able to produce artificially and which was illustrated in the publications just referred

²⁷ Babes, V., *Z. Hyg. u. Infektionskrankh.*, 1895, 20, 412.

²⁸ Churchman, J. W., *J. Exp. Med.*, 1927, 46, 1007; 1929, 18, 413.

scrutinized and if any "diminution of size" can be detected the apparent capsules are brushed aside as "retraction spaces or zones."

Outside the somata as just defined, and usually invisible save after the use of special methods of staining, other structures known as the "capsules" are demonstrable in a group of organisms known as the "capsulated group." The chapter of bacteriology dealing with these structures is marred by a good deal of confusion; but there is at least one widely studied organism which possesses a capsule so readily stained, and in such a variety of ways, so constant in its site and general behavior, that about it there is quite universal agreement. This organism is *D. pneumoniae* Type III S. One is justified in regarding it as typical of the capsular group and in defining the capsule as a structure (like that found on the pneumococcus) which surrounds the bacterial body; which is not stained by the ordinary bacteriological dyes; which is occasionally stained by Gram but as a rule only by other and more special methods involving the use of mordants; which sometimes appears as a clear unstained area standing out against a stained background (negative staining); which seems to possess a confining membrane, and which often enlarges on contact with the animal body. The structures found about the somata of other well established "capsulated" organisms—like *B. anthracis*, *Klebsiella pneumoniae* Friedländer and *Clostridium welchii*—conform to this definition. Indeed *B. friedländeri* might perhaps be taken as the type organism with even more justification than *D. pneumoniae* since its capsule is typical and since it was the first pathogenic organism on which this structure was demonstrated. Abel¹ does so regard it. If, then, we are to speak of a capsule at all, it is inevitable that we take the capsules of *D. pneumoniae* and these other orthodox species as standards. Otherwise the term is meaningless.

When stained by our method the R and the S forms of *D. pneumoniae* (Types I, II and III) appear just alike. The soma of each is stained blue; and usually (variations in result have been mentioned under Criticism of the technic) around each is a pink ring which occupies exactly the position of the margin of the capsule of the S

¹ Abel, R., in Kolle, W., and von Wassermann, A., *Handbuch der pathogenen Mikroorganismen*, Jena, Gustav Fischer, 3d edition. (Kolle, W., Kraus R., and Schuth, P.), 1929, 6, pt. 1, Liefg. 19, 243.

structure. Though not necessarily dependent on contact with the animal body for "development" its size may be increased by residence in living tissues and probably also by growth on media of proper constitution. When enlarged in these or other ways, capsules not otherwise easily made out may become clearly visible. Structures thus capable of swelling may also shrink; and capsular size is certainly inconstant. In one and the same specimen, large and small capsules are often seen (Fig. 2) just as are large and small somata.

Capsules vary also in their staining reactions. There is a widely held belief that this ought not to be expected to be the case; that all capsules should be supposed beforehand to react alike toward dyes; that if a capsule is not demonstrable on an organism by a technic which makes its demonstration possible on *another* organism it must necessarily be absent from the former. No such idea obtains concerning bacterial somata. No one is surprised that some of these retain the stain in Gram's technic, while others give it up; nor that some are acid-fast and others not. The expectation of absolutely constant staining behavior on the part of the capsules does not appear to be well founded. One property the majority of capsules have in common; they often—indeed usually—remain unstained by dyes which stain somata readily, unless these dyes are used in conjunction with mordants or after some special method of fixation. Among themselves, however, capsules vary in facility of taking or retaining stains and on the basis of this characteristic they may be graded as follows:

Group 1. Those which stain with relative ease, as by the Gram, the Friedländer or the Johne methods. Here belong for example some strains of *D. pneumoniae* III S, of *Klebsiella* Friedländer and of *B. anthracis* (from the animal body).

Group 2. Those which stain only by the well known capsule methods. Here belong the majority of strains of "capsulated" bacteria.

Group 3. Those "non-capsulated" bacteria on which capsules have not up to this time been regularly demonstrable but on which they have been observed by special technics (like the method of Cooper) and under somewhat special conditions of growth or environment. Here belong certainly some strains of the *coli-typhi-enteritidis* group and perhaps a number of others.

of as bacterial body; ectoplasm is that part usually spoken of as capsule, together (when these exist) with flagella.

In his first communication Toenniessen, studying *B. friedlaenderi*, used the term "ectoplasm" just as Zettnow had done. It was at that time, for him, synonymous with capsule.

In his second publication the term "*Schleimhülle*" was introduced as a name for the broad zone sometimes, but not always, seen about *B. friedlaenderi*. This was now described by him as the capsule, or as part of it.

In his third publication he stated: "Cell protoplasm and cell membrane would be better terms than endoplasm and ectoplasm. . . . The bacterial body consists of 1. Endoplasm (nuclear and plasmal material mixed). 2. Ectoplasm, a '*Hülle*' about the endoplasm; and 3. A broad sharply limited layer outside the ectoplasm; an excretion product; the '*Schleimhülle*' or '*Gallerthülle*;' the so called capsule. . . . Not a vital part of the real bacterial body but of great biological significance."

In his fourth publication he stated: "The term 'capsule' is not quite exact, for that which—by the usual method for demonstration of capsules—surrounds like a capsule the dark stained endoplasm consists of ectoplasm and the broad '*Aussenhülle*' together. Only the latter should in my opinion be spoken of as a capsule. It is formed by excretion from the ectoplasm, and between it and its surroundings there is no further peripheral excluding membrane."

It is clear that Toenniessen is by this time using Zettnow's terms in anything but their original sense. Capsule had at first been identical with ectoplasm; now it is nothing of the kind, but lies quite outside it, is indeed secreted by it. It is equally clear that if capsule be defined as Toenniessen came to define it neither *B. anthracis* (from the mouse's spleen), nor *Clostridium welchii*, is capsulated; for neither of these organisms possesses necessarily an *Aussenhülle*. Nor are the majority of strains of *D. pneumoniae* III S capsulated, for it is well known that the *Aussenhülle* is rarely, not constantly demonstrable about these organisms, even when structures which every bacteriologist would consider typical capsules are clearly stained. There appears to us no more reason for regarding secretory products as part of the capsule than for regarding sweat as a structural part of the skin.

A similar confusion runs through the other literature, making itself evident in the large numbers of ill defined terms employed. As a matter of fact the notion that an organism cannot be capsulated unless capsules are demonstrable by methods now available, is so firmly fixed in the minds of many bacteriologists that when capsular structures are, by new methods, demonstrated on "non-capsulated" bacteria the first impulse usually is to resort to ambiguous terms to describe them, even though these structures appear to be identical with the capsules of "capsulated" forms. Some of the terms thus used are ectoplasm, retraction space, swollen bacterial membrane, aureole, pseudocapsule, clear zone, halo, etc. Sometimes the attempt to make sharp structural distinctions is abandoned and everything—except soma—is grouped together as the "outer functional element." More

6. It has been impossible to produce with our staining methods structures resembling capsular membranes around collodion particles, red or white blood cells, or *Rickettsia* bodies. About red blood cells, it is true, the stain occasionally retracts, leaving a space; but this appearance is not usual and even when it occurs the space is never surrounded by a stained line.

7. Capsules have been demonstrated about some organisms (that is *B. subtilis* and *Bact. granulosis*) also by Casares Gil stain. One would have, therefore, to imagine identical artifacts produced by two different dyes.

8. There appears to be no relation between the size of the soma and the size of the capsule. This would be expected to be the case if the apparent capsular membrane were simply a deposit of stain.

The evidence concerning the nature of these structures would, of course, be more satisfactory if one could stain the capsular substance as often as one stains the capsular membrane, instead of only occasionally. On the other hand, it is perfectly well known (as a glance at the illustrations in any text-book of bacteriology will show) that the capsules of "capsulated" organisms frequently stain only at the periphery, leaving the capsular substance quite clear (see Fig. 8 as well as some of the individuals in Fig. 2). In any event, one must in some way account for these clear spaces; they are not vacua.

Whatever the nature of the structures we have described we do not wish to be understood as suggesting that bacterial capsules necessarily resemble each other in any way. That they differ in chemical and biological properties appears certain. It is also known that they differ in the same organism under varying circumstances (*e.g.* the change produced in the capsule of *D. pneumoniae* III S by injection into the peritoneum of the mouse). It is to be expected that they will be found to differ in physical characteristics. The evidence we have presented should, we think, only be interpreted as justifying a re-examination of the whole question in which the problems raised will be attacked by means of methods not subject to some of the limitations of tinctorial technic. Such studies we now have under way.

SUMMARY

A new method for staining capsules is described. With this method appearances are produced about the somata of a number of non-capsulated organisms which resemble capsules.

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FIG. 29. *B. anthracis* from agar; note halo.

FIG. 30. *Klebsiella* Friedländer; soma, capsular substance, capsular membrane and *Aussenhiülle*.

FIG. 31. *Escherichia coli* Th. Smith; soma, capsular membrane and flagella stained.

FIG. 32. Empty capsule (*B. subtilis*). Note attachment of flagella to capsular membrane.

FIG. 33. Empty capsule (*B. subtilis*). Note soma tipped out of capsule whose membrane is sharply stained; capsular attachment of flagella clearly shown.

FIG. 34. Two chains of *Streptococcus faecalis* showing the 4 individual cocci at the right (in both organisms) normally placed within the capsule, and 2 individual cocci at the left tipped out of the capsule.

FIG. 35. *B. subtilis*. Note in the upper organism the soma lies at the side of the capsule and that 2 flagella appear to be attached to it. Note the halos around the flagella and also around the capsule.

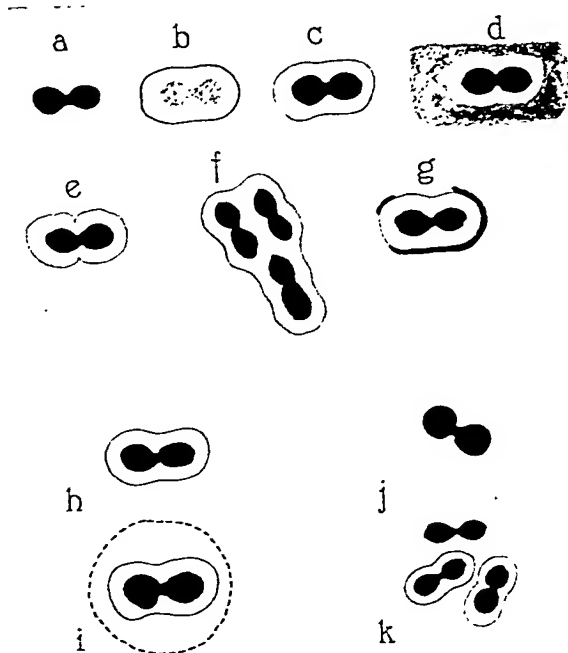
FIG. 36. (a) 4 individuals from a smear of *Eberthella typhi*. Apparent somatic attachment of flagella in individual at upper left hand corner in which no distinction between soma and capsule is to be made out. Structure of organism clear in other 3 individuals, 2 of which are flagellated and show definitely that the flagella are actually attached not to the soma but to the capsule. (b) *Bact. granulosis* showing apparent somatic attachment of flagella; no distinction between soma and capsule to be made out.

FIG. 37. *B. anthracis* stained by modified Burke method (short staining, long decolorization). The appearance as of a central Gram-negative medulla covered here, entirely, and there, by plaques and granules of Gram-positive material is clearly to be made out. Note halo.

FIG. 38. A thermophile stained by Burke's method. The individual at the upper left hand corner is provided with a Gram-positive cortex which covers the entire organism. In other individuals the cortex appears to be only partly developed, partially covering the Gram-negative medulla with plaques and granules of Gram-positive material.

FIG. 39. *B. anthracis* from spleen of mouse, stained by modified Burke's method and then with aqueous eosin. All three layers of the organism (capsule, cortex and medulla) are clearly seen. At the left is a long individual with several segments, all of which are thick and black, *i.e.* definitely Gram-positive; its capsular membrane is sharply seen; and between soma and membrane is the nearly clear space of the capsular substance. Immediately to the right of this organism is one whose soma consists entirely of a thin Gram-negative medulla with no Gram-positive cortex whatever; its capsule is sharply defined. In the other individuals are to be seen a number of combinations of these two pictures.

evidence that flagella and somata are chemically unlike is furnished by Craigie's uranium crystal violet technic²⁵ in which the bacterial bodies are stained dark



TEXT-FIG. 1. (a) Organism stained by methylene blue alone; soma, no capsule. (b) Organism stained by our technic; soma, black; capsular substance, grey; capsular membrane, delicate black line. (c) Same as b except that stain has been washed from capsular substance which appears as a clear space. (d) Negative "staining;" capsular substance appears as a clear space against a stained background, and a stained soma. (e) Same as c except that capsular membrane follows the indentations of the diplococcus. (f) Several diplococci within one capsule (fusion of capsules). (g) Precipitated stain lying on the capsular membrane. (h) Black soma; unstained capsular substance; black line of capsular membrane; greyish outer zone or *Lusenhülle*, merging into the stained (greyish) background. (i) Same as h except that outer zone is demarcated by concentration of stain at its periphery (dotted line). (j) No capsule stained; soma, black; outer zone, a clear space. (k) Two diplococci with capsule, and one without, lying within a single outer zone.

²⁵ Craigie, J., *J. Roy. Micro. Soc.*, 1929, 49, 9.

FIG. 29. *B. anthracis* from agar; note halo.

FIG. 30. *Klebsiella* Friedländer; soma, capsular substance, capsular membrane and *Aussenhiülle*.

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convincing proof that emptying of the capsule does occur. It is certainly striking that no sign of injury, at the site of extrusion of the organism, is to be seen in these instances.

The flagella attached to the portion of the capsule nearest the observer's eye may appear to be attached to the bacterial body; but up-and-down focussing will show that this is not the case and will reveal them arching from the near or distant surface of the capsule and not coming in contact at all with the bacterial body. In the photograph shown in Fig. 35, for example, the flagella on the left side of the upper organism appear, at first glance, to be attached to the bacterial body, which lies (in this instance) at the side of the capsule. In the examination of the specimen itself, however, careful focussing showed quite clearly that this was not the case. In no instance in which capsular membrane and capsular substance were clearly demonstrable have we seen flagella piercing the membrane and coming into contact with the bacterial body.

In some instances, however, (*e.g.* the bacterium represented in the upper left hand corner of Fig. 36 *a*, which possesses 3 well defined flagella; or the 3 monotrichous organisms pictured in Fig. 36 *b*) no capsular membrane, capsular substance or soma—as such—is to be made out. The reason for this doubtless is that all parts of the bacteria are deeply stained and the structure is thus obscured. This accounts for the large apparent size of such bacteria; one is really looking not (as is usually assumed) at the soma alone—with its attached flagella—but at soma plus capsule. Attention has been called to this appearance by others who have studied capsular staining but its significance has been overlooked. In these instances, too, despite the apparent somatic attachment of the flagella, it is certain that—at least in many cases—they are actually attached only to the capsule. That this is the case becomes clear from examination of the 3 other organisms depicted in Fig. 36 *a*. These lay in different fields of the same specimen from the one which contained the organism shown in the upper left hand corner. In one individual, soma and capsule are readily seen, but no flagella have been stained; in another (lower right hand corner), soma and 1 flagellum, attached to the capsule, are stained; while the third shows a soma and a number of flagella attached to the capsule. That what appears to be a large bacterial

to, occurs also in some bacteria in their native state. We have observed it particularly in thermophiles one of which—it grew at 52°C. but was not further identified—is shown in Fig. 38. The film photographed was made from a young culture and was stained by Burke's method. Here and there is a thick organism, entirely Gram-positive. But the majority of vegetative forms are patchily stained as though made up of a Gram-negative rod, surrounded by granules or plaques of Gram-positive material. It would appear that in these individuals either a complete Gram-positive cortex never develops or that the cortex, if fully developed, never attains to homogeneity of chemical composition, that is to say, of behavior toward the stain.

The relation of capsule to cortex was not until recently clear, for in the specimens studied by the method used in the earlier experiments, in which medulla and cortex were first observed (Burke's method), the capsules were not stained. Recently, however, we have, by the following technic, succeeded in staining differentially all three layers: capsule, cortex and medulla.

A film was made from an aqueous suspension of the spleen of a mouse dead of anthrax. This was stained by a modified Burke method (exposure for a few seconds to methyl violet and sodium carbonate; stain washed off; exposure to iodine for a few seconds; acetone-ether decolorization for 5 minutes). Examination at this stage showed beautiful differential staining; thick, bluish, Gram-positive segments and slender pink Gram-negative segments were to be seen. The smear was then exposed to 1 per cent aqueous eosin, which stained the capsular membrane pink.

Now all three layers were to be seen. Lying within the capsule, and separated from the membrane by a wide space, were the somata. Some of the segments were normal in size and bluish black (*i.e.*, Gram-positive). In others, the Gram-positive cortices had been decolorized or destroyed; and these were much slenderer than normal and stained pink (*i.e.*, Gram-negative). In some parts of the smear, all of the segments were Gram-positive; in others, they were all Gram-negative. Not infrequently, a capsule contained one Gram-positive and one Gram-negative segment (Fig. 39).

Nature of the Capsule

The capsule as revealed by staining methods thus appears to be a frequent, perhaps a constant—certainly not an occasional—bacterial

Group 4. Those which have thus far been stained only or best by the methods described in this communication.

Critique

Are the structures here described as seen about organisms usually regarded as non-capsulated really capsules? That the method used does stain the capsules of "capsulated" organisms is open to no doubt; nor is there any doubt about the fact that the structures often seen with this stain about the "non-capsulated" group are quite similar in site, configuration and relative size to the well recognized capsules. We have studiously avoided speaking of them as "pseudocapsules," because the term appears to us a meaningless evasion. That they are not artifacts appears to us pretty well established by the following considerations:—

1. The capsular membrane has a definite configuration and follows the indentations of diplococci, as well as the partitions of organisms in fission.
2. The bacterial bodies often occupy an eccentric position in the apparent capsular cavities and occasionally one segment of a chain lies at one side of the capsular cavity, another segment at the other side. This they would hardly do if the stain were simply deposited about them, as about particulate bodies.
3. The bacteria in a chain may be very irregularly arranged without affecting the regular configuration of the capsular membrane (see Fig. 3). This again is hardly the picture of dye simply deposited about a small body.
4. The capsular membrane is often differentially stained. This is not what would be expected if it were caused simply by precipitation of Wright's stain.
5. When the bacterial bodies are dislodged from their apparent capsules (in making the film) no capsular membrane ever appears about these bacterial bodies in their new positions, as would be expected if the appearances described were due simply to agglomeration of stain about microscopic bodies. On the contrary, what appear very definitely to be empty capsules are often seen (occasionally with flagella attached to them); and these are not infrequently obviously at the previous site of the dislodged organisms.

EXPLANATION OF PLATES

All photomicrographs made at $\times 2000$ magnification. Unless otherwise stated the stain used was Wright's, which colored the bacterial bodies blue, the capsular membrane pink or pinkish purple.

PLATE 33

FIG. 1. *D. pneumoniae* III R from blood agar.

FIG. 2. *Klebsiella* Friedländer; note all varieties of staining from deep staining (no differentiation of structure) to ideal staining (structure perfectly shown).

FIG. 3. *B. anthracis* from spleen of mouse.

FIG. 4. *B. anthracis* from agar.

FIG. 5. *Clostridium tetani*.

FIG. 6. *Alcaligenes faecalis*.

FIG. 7. *D. pneumoniae* III S.

FIG. 8. *D. pneumoniae*; negative "staining" of capsule.

FIG. 9. *Klebsiella* Friedländer; deep staining of soma and capsule; *Aussenhülle* clearly seen.

FIG. 10. (a) *B. subtilis*. (b) *Eberthella typhi*; at left, a group with fused capsular membranes and 2 flagella; at right, a single organism with capsule and no flagella.

FIG. 11. *B. subtilis*. Upper organism; soma and capsular membrane sharply stained; capsular substance clear. Lower organism; soma tipped out of capsule; secondary zone outside capsule (*Aussenhülle*).

FIG. 12. *Bact. granulosus* S.

PLATE 34

FIG. 13. *Escherichia coli*, MacNeal stain.

FIG. 14. *B. anthracis*, Giemsa stain.

FIG. 15. *B. subtilis*, Casares Gil stain; stumps of flagella attached to capsule.

FIG. 16. *D. pneumoniae* III S from peritoneum of mouse.

FIG. 17. *D. pneumoniae* I R from blood agar.

FIG. 18. *D. pneumoniae* II R from blood agar.

FIG. 19. *Serratia marcescens*.

FIG. 20. *Pseudomonas aeruginosa*.

FIG. 21. *B. subtilis* var. Chester.

FIG. 22. *Erysipelothrix muriseptica*.

FIG. 23. (a) Scratched line on a Leitz ruled slide. (b) *Eberthella typhi*.

FIG. 24. *Bact. granulosus* R.

PLATE 35

FIG. 25. *Proteus vulgaris*.

FIG. 26. *Streptococcus faecalis*.

FIG. 27. *Mycobacterium tuberculosis* *tuberculosis*.

FIG. 28. *Mycobacterium tuberculosis* *tuberculosis*, Casares Gil stain.

the conclusions seem unavoidable that vitamin A is essential in most vertebrate species and that its withdrawal is followed by a common effect upon epithelial structures. For convenience this change will be called metaplasia regardless of exact connotations of the word.

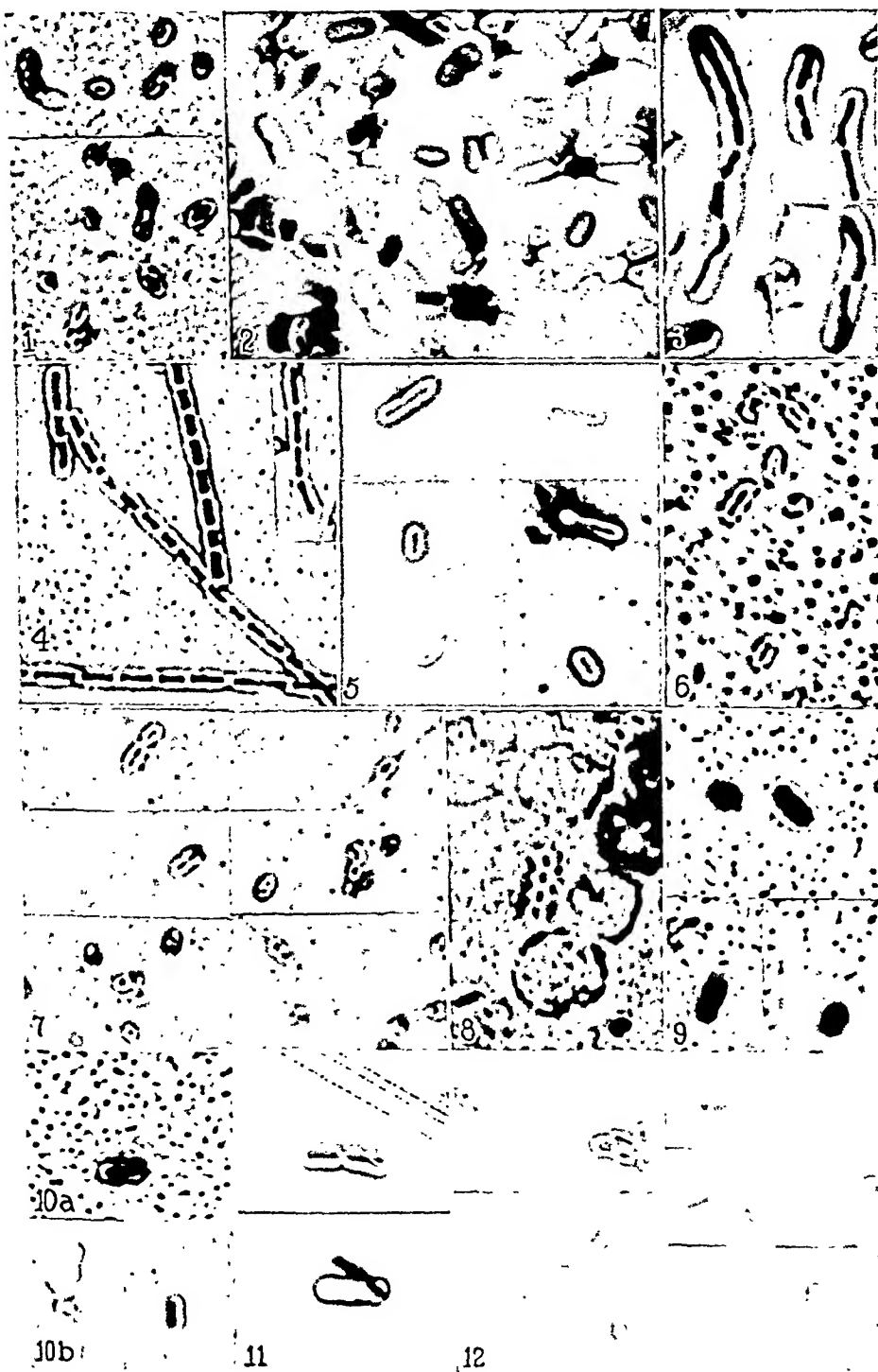
Our conception is that the absence of vitamin A creates a starvation specific for many epitheliums, resulting in atrophy, which in turn stimulates reparative proliferation of basal cells which alone are capable of multiplying in epitheliums with secretory functions. The new (reparative) cells which replace the old in all locations and regardless of previous function and morphology acquire a common form and arrangement—that of a stratified keratinizing epithelium (Fig. 9). One is tempted to say that the cells concerned in repair revert to a common primitive type but is restrained by the unprovable if reasonable ontological implications involved. The replacement epithelium in any location is identical with that in all others. The morphology warrants conviction of absence of secretory function. Cells of varied functions and morphology are completely masked by common features in A deficiency.

Early experiments indicated that restoration of vitamin A to animals far advanced in the deficiency resulted in the return of epitheliums normal for each situation.

It is the purpose of this report to describe the sequences of repair in white rats in epitheliums only. Simple as this project seemed it proved difficult because of the variation in order of organs involved as the deficiency progressed as was pointed out by us in 1925. Fortunately our series of complete histological studies of 72 A-deficient rats was sufficiently large to enable us to predict the probabilities as to histological changes (metaplasia) in rats used for repair experiments after consideration of weight changes and duration on the deficient diet. Gross observations in post mortems of more than 200 other A-deficient rats gave us additional evidence for our estimations of progress of metaplasia because the changes in appearance when the keratinization is fully developed are visible to the naked eye in many locations.

Methods

The diet was that employed by us in 1925. This diet is deficient also in vitamins C, D and E. We have adhered to it because (1) evi-



different durations of deficiency in rats studied histologically. Because in many of the animals equally marked metaplasia was present in two organ groups, the figure $\frac{1}{2}$ is used in the proper columns to indicate such distributions. The figure 1 is used where one organ group was outstandingly affected. It should be remembered that in every rat beyond the 50 to 60 day period some organ of all groups was affected as well as organs not included in Table I as thymus, pancreas and thyroid.

The last horizontal row in Table I under Miscellaneous includes a

TABLE I

Duration	Examples	Ocular-para-ocular	Salivary gland group	Respiratory tract	Genito-urinary tract	Negative
<i>days</i>						
50-60	6	$\frac{1}{2}$	$\frac{1}{2}\frac{1}{2}$	$1\frac{1}{2}$	$\frac{1}{2}\frac{1}{2}$	11
60-70	6	$\frac{1}{2}$	$11\frac{1}{2}\frac{1}{2}$	$11\frac{1}{2}$		
70-80	4	$1\frac{1}{2}$	$11\frac{1}{2}$			
80-90	4	$1\frac{1}{2}$	$1\frac{1}{2}\frac{1}{2}$	$\frac{1}{2}$		
90-100	10	$\frac{1}{2}$	$11\frac{1}{2}\frac{1}{2}\frac{1}{2}\frac{1}{2}$	11	$1\frac{1}{2}\frac{1}{2}\frac{1}{2}\frac{1}{2}$	
100-110	2		$1\frac{1}{2}$	$\frac{1}{2}$		
130-150	6		$\frac{1}{2}\frac{1}{2}\frac{1}{2}$	$\frac{1}{2}\frac{1}{2}\frac{1}{2}$	$11\frac{1}{2}\frac{1}{2}$	
150-170	8		$1\frac{1}{2}\frac{1}{2}\frac{1}{2}$	11	$11\frac{1}{2}\frac{1}{2}\frac{1}{2}$	
170-190	9	$\frac{1}{2}$	$\frac{1}{2}\frac{1}{2}\frac{1}{2}\frac{1}{2}$	$111\frac{1}{2}\frac{1}{2}\frac{1}{2}\frac{1}{2}$	$\frac{1}{2}\frac{1}{2}\frac{1}{2}$	
210-230	1		$\frac{1}{2}$	$\frac{1}{2}$		
236-260	3		$\frac{1}{2}$	1	$1\frac{1}{2}$	
Miscellaneous	8	$\frac{1}{2}\frac{1}{2}\frac{1}{2}\frac{1}{2}$	$\frac{1}{2}\frac{1}{2}\frac{1}{2}$	$1\frac{1}{2}\frac{1}{2}$	$11\frac{1}{2}$	
Total.....		7	$22\frac{1}{2}$	21	$16\frac{1}{2}$	2

number of rats which for brief periods received butter fat at times when it seemed apparent that they could no longer survive the deficiency and were thus carried for periods of 8 months to more than a year.

If we assume that at any given period the maximum metaplasia is found in the regions first responding to the deficiency, Table I practically substantiates the impression we have obtained throughout our histological studies that the order of response by metaplasia in the rat is salivary glands, respiratory tract, genito-urinary tract and lastly the ocular-paraocular group.

Contrary to prevailing impressions, eye symptoms are a late effect and this has repeatedly proven true in the study of human material.

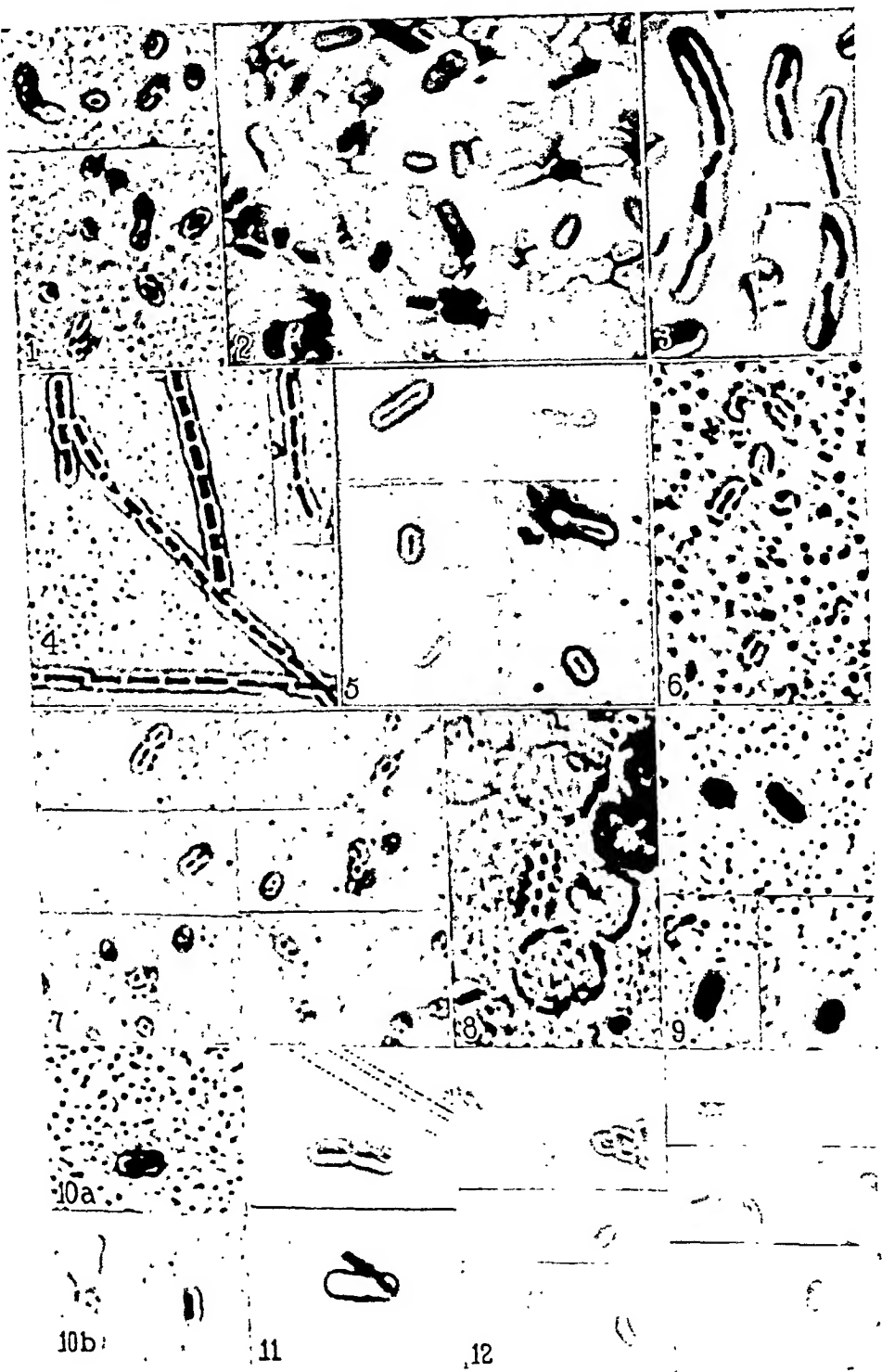


TABLE II
Thirty-five Recovery Experiments
Vitamin A Deficiency Rats

Rat No.	Sex	Duration of deficiency diet	Duration of treatment
		days	days
x338	♀	53	19
218	♀	62	21
x340	♀	63	8
x334	♂	64	5
212	♂	70	9
239	♂	103	5
233	♀	114	3
234	♀	114	6
425	♀	118	10
232	♂	137	6
235	♀	138	5
397	♀	138	12
403	♂	138	12
393	♂	143	18
394	♀	146	22
405	♂	150	10
413	♀	150	13
399	♀	151	15
400	♀	151	20
396	♀	160	6
401	♀	161	22
409	♂	170	13
411	♀	170	13
412	♀	170	14
415	♀	170	14
414	♀	170	19
417	♀	187	7
418	♀	187	8
50	♀	185	43
x354	♀	196	4
x343	♂	221	3
x345	♀	248	4
x341	♂	248	4

x indicates that a cod liver oil concentrate instead of butter fat was used to induce recovery.

of bacteria, but even then extension through the living epithelial lining into adjacent tissues was very rare. A few instances of impor-



In addition to resumption of growth in bones and teeth and the restoration of proper epitheliums, other important changes in A deficiency repair occur in bone marrow and spleen, essentially hyperplasias of erythroblastic elements.

The early surveys of the histology of the rats in stages of recovery were confusing because of apparent lack of sequence and variation in findings in the different organs of individual rats. After a thorough review study of the deficiency changes in untreated rats our difficulties were cleared away by realizing that we could estimate in any recovering individual rat the stage or degree of metaplasia which existed before treatment. We shall omit many histological details which can be arranged in sets or periods of recovery phenomena, initiated at different stages of progress in developing metaplasia.

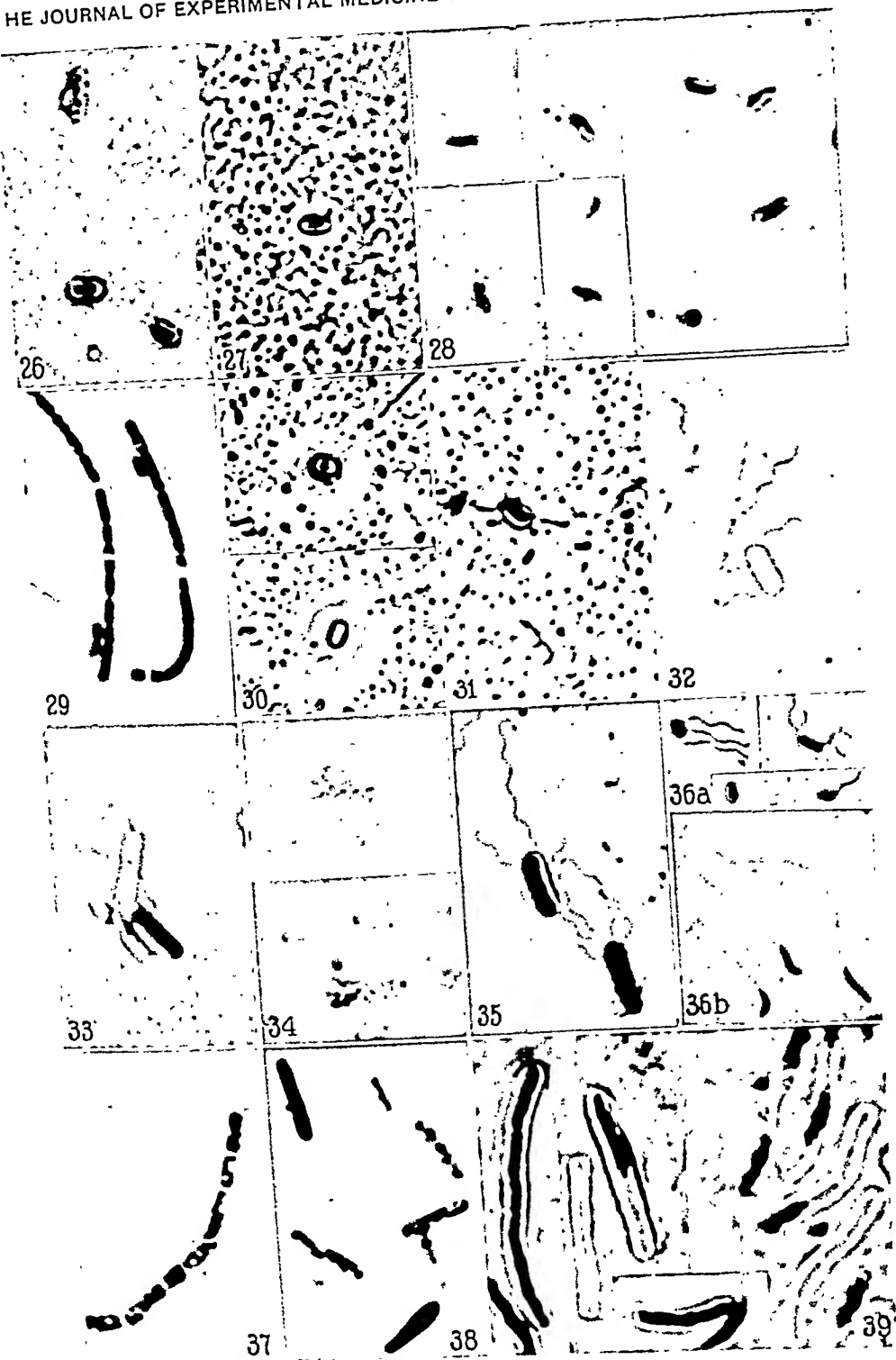
The observations of major interest can be considered under three headings:

1. Repair initiated before metaplasia is complete at the stage when the original epithelium, more or less atrophic, is undermined by an epithelium of basal cell origin which has not had time to develop into a stratified or keratinizing epithelium.

2. Repair initiated after metaplasia is complete when the original epithelium has been replaced by a stratified squamous keratinizing epithelium comparable layer by layer with the epidermis.

3. Repair of epitheliums normally either stratified or of the transitional type which, as a result of A deficiency, have become hyperkeratotic, such as the conjunctivae and bladder.

Repair Following Incomplete Metaplasia.—Now we must recall the fact that frequently in the progress of A deficiency an atrophic epithelium is underlaid by a layer of multiplying cells one or more deep and as yet not differentiated to a recognizable degree. This process of undermining apparently takes place rapidly and the replacement cells may consist of a single layer of very flat cells resembling peritoneal or pleural cells rather than cells with epidermoid potentialities. In some instances the maximum surface has been covered for the number of cells now replacing the atrophic original epithelium still *in situ*. Whether the replacement layer is one or several cells deep, each cell is capable of differentiating into the type normal for its situation, as well as into the keratinizing type. The effects of vitamin A adminis-



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EPITHELIAL REPAIR IN RECOVERY FROM VITAMIN A DEFICIENCY

AN EXPERIMENTAL STUDY

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PLATES 36 TO 38

(Received for publication, October 26, 1932)

The earliest demonstrable effect of vitamin A deficiency in rats¹ and guinea pigs² is upon epithelial structures.

The sequences are atrophy of the epithelium concerned and the substitution for it of a stratified keratinizing epithelium identical in appearances in all locations and arising from *focal proliferation of basal cells*. Since replacement by keratinizing epithelium in many organs has also been found in human infants,³ in the monkey *Macacus rhesus*,⁴ in the albino mouse,⁵ and gross changes point to the same phenomenon in swine,⁶ in dogs,⁷ in rabbits,⁸ in calves⁹ and in the domestic fowl,¹⁰⁻¹²

¹ Wolbach, S. B., and Howe, P. R., *J. Exp. Med.*, 1925, 43, 753.

² Wolbach, S. B., and Howe, P. R., *Arch. Path. and Lab. Med.*, 1928, 6, 239.

³ Wilson, J. R., and DuBois, R. O., *Am. J. Dis. Child.*, 1923, 26, 431. Also several other unpublished cases in the pathological service of the Children's and Infants' Hospitals of Boston.

⁴ Tilden, E. B., and Miller, E. G., *J. Nutrition*, 1930, 3, 121.

⁵ Wolfe, J. M., and Salter, H. P., Jr., *J. Nutrition*, 1931, 4, 185.

⁶ Hughes, J. S., Aubel, C. E., and Lienhardt, H. F., *Kansas State Agric. Col. Techn. Bull.*, No. 23, 1928.

⁷ Steenbock, H., Nelson, E. M., and Hart, E. B., *Am. J. Physiol.*, 1921, 58, 14.

⁸ Nelson, V. E., and Lamb, A. R., *Am. J. Physiol.*, 1920, 51, 509. Nelson, V. E., Lamb, A. R., and Heller, V. G., *Am. J. Physiol.*, 1922, 59, 335.

⁹ Jones, I. R., Eckles, C. H., and Palmer, L. S., *Dairy Sci.*, 1926, 9, 119.

¹⁰ Beach, J. R., *Science*, 1923, 58, 542.

¹¹ Guerrero, L. E., and Concepcion, I., *Philippine J. Sci.*, 1920, 17, 99.

¹² Emmett, A. D., and Peacock, G., *J. Biol. Chem.*, 1923, 55, 679.

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dences of C deficiency—scorbutic effects—cannot be produced in rats, (2) vitamin D is not necessary to prevent rickets in rats under proper hygiene and with proper inorganic elements in their diets, (3) the consequences of vitamin E deficiency have been accurately determined by Evans and do not concern the type of lesion we are dealing with and (4) we have found that histological recovery ensues when vitamin C only is restored to the diet. The plan of the experiment was to restore vitamin A, usually in the form of butter fat, to the diet of rats in various stages of the deficiency.

Our attempts to show graphically the order of appearance and degree of metaplasia in the various organs have been unsatisfactory because of our inability to devise quantitative expression of histological appearances.

Incidence of the Metaplasias

Sixty-six rats kept on the A-deficient diet for 60 days and longer all showed the keratinizing metaplasia in some part of the eye group, the salivary gland group, the respiratory tract and the genito-urinary tract. Of six rats on the diet between 50 and 60 days, two showed no metaplasia while four did to some extent in all of the four tracts mentioned above.¹³ Much study of a table obtained by recording the presence or absence of metaplasia in each organ for each rat yielded no information of value. The maximum and presumably the earliest effect during early periods centers about the salivary gland group and the respiratory tract. In later periods the genito-urinary tract became equally important. We are convinced that the conspicuous gross effects upon the eye are in large part due to accumulation of desquamated cells in the conjunctival sac and consequent irritation. Table I is an attempt to show the maximum distribution of the metaplasia for

¹³ Under the eye group we include the conjunctiva, Harderian, intra- and extra-orbital lacrymal glands and the Meibomian glands.

Under the salivary gland group—the submaxillary gland, the parotid gland and all accessory glands of the tongue, buccal cavity and pharynx.

Under respiratory tract—the nares, maxillary sinuses, Jacobson's organ, trachea and bronchi.

Under genito-urinary tract—the renal pelvis, ureters, bladder and the epididymis, prostate, seminal vesicles, coagulating glands, uterus, oviducts and accessory sex glands of the vulva.

restoration of vitamin A to the diet. They may not take place until 7 days or even later according to the condition of the rat. All rats which survived for as long as 9 days after treatment was started showed evidences of repair towards restoration of normal epitheliums but, as stated above, increase of weight is the only positive sign of effect of treatment to be correlated with epithelial repair. The response of the metaplastic epithelium is not simultaneous in all organs so that in any animal treated for periods longer than 10 to 12 days all stages of repair may be found.

The initial changes are separation of superficial keratinized cells and vacuolization of cells of intermediate layers. Polymorphonuclear leucocytes enter the epithelium and are found between cells and invading vacuolated cells. The vacuolization of cells seems to be the result of complete liquefaction or disappearance of cytoplasm. Nuclei of such cells rapidly become pyknotic and eventually disappear (Fig. 6).

Later, owing to the accumulation of leucocytes and increase of vacuolization, the epithelium often becomes divided into two zones, one of deep viable cells, presumably not irreversibly differentiated and an upper zone of cells which degenerate and die (Figs. 1, 4 and 8). Where the metaplasia has been so complete as to duplicate the strata found in epidermis, we are of the opinion that only the stratum germinativum survives.

In some instances a bottom layer two or three cells deep survives, but probably only in locations where there have been unusual collections of undifferentiated cells from basal cell proliferation.

The keratinized cells disappear in extraordinary fashion, impossible to follow, and permitting no explanation other than that of a lytic process. Our supposition was that the completely keratinized cells would remain in ducts for considerable periods during repair, very promptly, however, after the initial vacuolization of cells and leucocytic infiltration has taken place, the keratinized cells disappear, leaving only granular debris or a very delicate barely visible reticulum in the lumina of ducts. Phagocytosis plays no part in the repair process. No example was found in the intensive study of the rat in these experiments. Cells of intermediate layers corresponding to the stratum granulosum and rete mucosum may after vacuolization of adjacent cells of the same layers undergo a hyaline change and separate off, after which they disappear as do the keratinized cells.

(In twelve infants showing postmortem evidences of A deficiency, the maximum effect was in the respiratory tract; in extreme cases only were the eyes affected.)

The Repair Phenomena

Thirty-five rats were used in the study of the repair phenomena. The periods on deficient diet ranged from 53 to 248 days. The periods of treatment with butter fat ranged from 3 to 43 days. Our observations while covering all organs were chiefly directed to the salivary gland, the trachea and the uterus. Accordingly many more female rats were used than male. A few rats were given cod liver oil concentrate (indicated in Table II) as a means of restoring vitamin A.

We noted no qualitative difference in the epithelial responses, but as the concentrate was not calibrated any differences would have been without significance. On the whole, repair was more tardy with the cod liver oil concentrate than with butter fat.

The weights of all the rats were taken weekly. Comparisons of the increase of weight, following restoration of vitamin A, with the evidences of epithelial repair for each rat showed without question that the degree of repair was commensurate with the recovery in weight and not with the duration of treatment. Here again our inability to assign numerical values to degrees of presumable repair processes made attempts at charting results a futile performance. A clean-cut correlation with gain of weight in the repair period was found in the restoration of growth activities of bone at epiphyseal-diaphyseal junctions. Owing to the fact that a plate of bone forms on the diaphyseal side of the atrophic metaphyseal cartilage in advanced A deficiency, it is possible to compare and probably to measure the amount of growth of long bones during the recovery period. Bones and teeth in recovery from A deficiency will be discussed elsewhere.

Probably the most important factor in influencing recovery from A deficiency is the presence or absence of infection. Important infections were surprisingly rare in our animals. For example, death from pneumonia was a very rare occurrence. Local suppuration was not uncommon in and adjacent to cysts of unusual size distended with desquamated epithelium. Ducts distended with cornified cells and communicating with the mouth naturally afford a medium for growth

thelium persisted. This was true also in other glands where the parenchyma had atrophied beyond repair as in the lacrymal and Harderian glands. In infected cysts with purulent contents the keratinizing epithelium persists, notably in bronchiectatic cavities in the lungs. The longest period was in a rat kept for 185 days on the deficient diet followed by 43 days treatment with butter fat. This rat showed keratinizing epithelium in bronchiectatic cavities and in the cicatrized remains of the Harderian glands. In all other locations normal epithelium had returned although cicatrices in the salivary glands and lacrymal glands gave evidence of previous lesions with infection.

Repair of Epitheliums Normally Stratified.—This type of repair proved to be the simplest and applies to the first portion of the stomach where focal hyperkeratoses arise, to the conjunctiva, palpebral and corneal, the vagina and the urinary bladder. It was most easily followed in the corneal epithelium, palpebral conjunctiva and bladder.

Promptly after restoration of vitamin A to the diet there is complete desquamation of keratinized cells. The effect of this can be followed in the living animal in the rapid amelioration of signs of conjunctival irritation. Even where the duration and degree of hyperkeratosis of the cornea were such as to produce a vascularization of the cornea the return to the normal epithelium was prompt, 7 to 8 days, and complete. The blood vessels in the tunica propria persist for an undetermined period after the recovery of the epithelium.

In the palpebral conjunctiva, mucus-secreting cells make their appearance in the deeper layers of cells before the desquamation of the superficial cells is completed.

In the urinary bladder and vagina some vacuolization of cells precedes the desquamation of the superficial keratinized cells and in early stages of repair indicates the line between the several layers of superficial degenerating cells and the lower viable layer of cells. Cellular infiltration is slight or absent (Figs. 3 and 4). Repair is completed by the cells of the basal layer assuming the morphology and functions of the normal type of epithelium.

DISCUSSION

While no mention has been made of each organ in which A deficiency metaplasia takes place, the repair phenomena have been studied in

tant infection were encountered both in the large untreated series and in the thirty-five treated rats. These were chiefly infections of bronchiectases secondary to plugging of bronchioles with desquamated keratinized cells and abscesses of the base of the tongue following unusually large cysts produced by long continued accumulation of desquamated keratinized cells. That infections of the lungs were not more common is surprising in view of the fact that fairly early in the deficiency, the ciliated epithelium of the respiratory tract may disappear and that foreign particles—starch granules, for example, from the food—can occasionally be demonstrated in the alveoli of the lungs.

The conviction we expressed in 1925 that the condition of vitamin A deficiency does not increase susceptibility to infection by bacteria remains and is strongly reinforced by all subsequent experiences.

Since the repair phenomenon is accompanied by infiltration of the metaplastic epithelium and supporting connective tissue by wandering cells, we emphasize here that the metaplasia of A deficiency in rats, guinea pigs and human beings, does not excite an infiltrative reaction, no matter how long the deficiency has continued unless secondary infection has occurred. Bacteria and leucocytes may be present in great numbers in the superficial and desquamated keratinized cells without the deeper layers of the epithelium or supporting tissues becoming infiltrated by any type of wandering cell.

Early in the study of the repair it became evident that the return to the normal type of epithelium was fairly abrupt and that the sequences were difficult to follow. The rapidity of the process and its initial elusiveness we now know are due to the fact that in the stratified keratinizing epithelium of A deficiency, the lowermost layer of cells, as in the stratum germinativum of epidermis, is composed of cells all capable of proliferation. The return to the normal epithelium is therefore a diffuse process in marked contrast to the focal proliferative response in the deficiency. No reparative change in epithelial tissues, bones or teeth, was seen in any animal before a substantial gain in weight had occurred as a result of the restoration of vitamin A to the diet. The shortest period therefore has been 6 days, while several rats either failed to show any gain or gained very slightly in periods as long as 14 days. Most rats continued to decline in weight during the first few days after restoration of the diet.

and include the vacuolar degeneration, leucocytic infiltration and desquamation of cells we have described.¹⁴

Another viewpoint to be considered is that the degeneration of cells and leucocytic infiltration represent inflammatory responses to the presence of cells become foreign after restoration of normal conditions of nutrition.

As it is probable that in stratified epithelium the cells above the stratum germinativum are dependent upon the latter for sustenance, we may reason that all the events are secondary to the sudden shift in trend of differentiation in the cells of the stratum germinativum. We have described how the cells of this layer proceed to redifferentiate. Early in recovery they must therefore become an independent tissue unit, no longer contributing to overlying cells, and must act as a barrier between overlying cells and the supporting connective tissue with its blood vessels. If the degeneration of the upper cell layers is due to withdrawal of nutrition, autolysis might be regarded as a logical consequence and the infiltration as a response to the presence of products of autolysis. However, the cytological appearances are not in accord with this theory in explanation of the vacuolar degeneration and infiltration, as both occur before the appearance of the usual familiar evidences of cell degeneration caused by withdrawal of nutrition. On the whole, however, we are inclined to believe that the theory approximates the facts and that the unfamiliar appearances accompanying degeneration and death of the cells are due to latent or potential physiological properties of the cells suddenly released or activated by restoration of vitamin A.

The fact of greatest interest is the preservation by the cells of the stratum germinativum, of the identity of the original epithelium throughout the period of metaplasia. The evidence is conclusive that all the cells of this layer can assume the original functions and morphology without undergoing division when supplied with vitamin A. We may, therefore, conclude that the nuclear chromatin remains unaffected by the deficiency.

We believe that careful cytological studies as the deficiency progresses and during the recovery phenomena may yield interesting correlations between morphology and function. In 1925 we concluded

¹⁴ Long, J. A., and Evans, H. McL., *Mem. Univ. California*, No. 6, 1922.

tration at this stage are seen in the increase of vertical diameter of the cells. If the replacement layer is several cells deep, some of the uppermost cells degenerate and cells from below extend up between them, the surface by elongation of their vertical diameters (Fig. 5). The atrophic original epithelium may be cast off before the replacement cells have changed much in morphology, giving rise to what was at first a perplexing appearance of trachea or gland ducts lined by a shallow layer of flat cells.

If the replacement layer at the initiation of repair is several cells deep, many of the superficial cells become rounded in form, the nuclei pyknotic, the cytoplasm hyaline and acidophilic in staining reaction. These degenerated cells separate and are cast off (Fig. 1). As recovery progresses the deeper cells increase in vertical diameter and gradually assume the characteristics normal to the organ. Where the replacement cells are not flattened and more than one cell deep, the ensuing epithelium becomes crowded in appearance and individual, well differentiated cells continue to degenerate (Fig. 1).

Where the replacement cells are few in number for the area covered, they may become differentiated in function before assuming normal dimensions; for example, we frequently found lining the trachea in part or completely, cells, ciliated and mucus-secreting, with horizontal diameters more than twice the vertical. The first evidence of the return of ciliation is the presence of minute paired dots beneath the cuticular border of surface cells, often at a stage when these cells are flat in shape. Vacuoles of mucus also appear in flat surface cells with and without evidence of returning ciliation.

The sequences described above all occur between the 5th and 13th days after restoration of the diet. The important features to be emphasized are that all or practically all of the replacement cells are still able to differentiate in either direction toward the squamous keratinizing type or toward the normal type.

Repair Following Complete Metaplasia.—Repair at this stage involves the removal and disposal of keratinized cells and deeper cells irreversibly differentiating in this direction. In contrast to the original epithelium the replacement epithelium has a stratum germinativum every cell of which is capable of division by mitosis.

The first changes in repair may be seen as early as the 5th day after

final weight not recorded. Cross-sections of two ducts, each one probably previously distended with keratinized cells. Note the vacuolar degeneration of remaining cells, the absence of cornified cells, the leucocytic infiltration and the flattened cells of the basal layer. These ducts were in an atrophic cicatrizing region of the gland, a condition not accompanied by complete restoration of the duct epithelium which usually reaches only a low cuboidal type.

FIG. 3. Renal pelvis. Rat 401. Repair in epithelium originally of transitional type. The cornified cells have desquamated. Intermediate layers in stage of vacuolar degeneration.

PLATE 37

FIG. 4. Uterus, distal end of cervix. Rat 401. A more advanced stage of repair than in Fig. 1. The line of cleavage between surviving cells and degenerating cells clearly shown. The stroma is sparsely infiltrated with lymphoid cells, polymorphonuclear leucocytes and rarely a mast cell.

FIG. 5. Submaxillary gland duct. Rat 405. 150 days on deficient diet, 10 days on recovery diet. Recovery following incomplete metaplasia. There is slight vacuolar degeneration. Cells of basal layer in process of direct resumption of normal morphology.

FIG. 6. Uterus, body, distal third. Rat 401. For duration of diets and weights of rat, see above. Repair following complete metaplasia. In right half of figure an epithelial "pearl" is disappearing by vacuolar degeneration and probable heterolysis through agency of leucocytes. Elsewhere the cornified epithelium has desquamated, other superficial cells are separating from one another. The deeper layers show infiltration and vacuolar degeneration.

FIG. 7. Trachea. Rat 336. This rat was kept on a partially deficient diet for 161 days, followed by 81 days on a completely deficient diet. To induce recovery 13 intraperitoneal injections of cod liver oil concentrate were given in a period of 17 days. The rat died after a slight gain in weight apparently from toxic effects of the concentrate. The trachea shows repair following probable incomplete metaplasia. There is evidence of desquamation of superficial cells. Cells of intermediate layers are surviving. There is considerable infiltration with mononuclear and polymorphonuclear cells. Infection cannot be ruled out as an explanation of the infiltration in the connective tissue.

PLATE 38

FIG. 8. Sublingual salivary gland duct. Rat 393. 143 days on deficient diet, 18 days on recovery diet. Initial weight 85 gm. Maximum weight 128 gm. At end of deficiency diet, 109 gm. On the recovery diet it gained 16 gm. in 12 days; final weight not recorded. Cross-section of a duct showing repair after complete metaplasia. Note columnar basal cells, infiltration and extensive vacuolar degeneration.

FIG. 9. Submaxillary gland duct. Rat 100. 181 days on deficient diet. To show complete metaplasia before the epithelium has become distorted by pressure of accumulated desquamated cells.

tration at this stage are seen in the increase of vertical diameter of the cells. If the replacement layer is several cells deep, some of the uppermost cells degenerate and cells from below extend up between them to the surface by elongation of their vertical diameters (Fig. 5). The atrophic original epithelium may be cast off before the replacement cells have changed much in morphology, giving rise to what was at first a perplexing appearance of trachea or gland ducts lined by a shallow layer of flat cells.

If the replacement layer at the initiation of repair is several cells deep, many of the superficial cells become rounded in form, the nuclei pyknotic, the cytoplasm hyaline and acidophilic in staining reaction. These degenerated cells separate and are cast off (Fig. 1). As recovery progresses the deeper cells increase in vertical diameter and gradually assume the characteristics normal to the organ. Where the replacement cells are not flattened and more than one cell deep, the ensuing epithelium becomes crowded in appearance and individual, well differentiated cells continue to degenerate (Fig. 1).

Where the replacement cells are few in number for the area covered, they may become differentiated in function before assuming normal dimensions; for example, we frequently found lining the trachea in part or completely, cells, ciliated and mucus-secreting, with horizontal diameters more than twice the vertical. The first evidence of the return of ciliation is the presence of minute paired dots beneath the cuticular border of surface cells, often at a stage when these cells are flat in shape. Vacuoles of mucus also appear in flat surface cells with and without evidence of returning ciliation.

The sequences described above all occur between the 5th and 13th days after restoration of the diet. The important features to be emphasized are that all or practically all of the replacement cells are still able to differentiate in either direction toward the squamous keratinizing type or toward the normal type.

Repair Following Complete Metaplasia.—Repair at this stage involves the removal and disposal of keratinized cells and deeper cells irreversibly differentiating in this direction. In contrast to the original epithelium the replacement epithelium has a stratum germinativum every cell of which is capable of division by mitosis.

The first changes in repair may be seen as early as the 5th day after

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The above described events take place with varying rapidity. In the trachea the vacuolar degeneration and leucocytic infiltration may be so extensive as to give the appearance of actual disruption of the epithelium closely simulating an inflammatory reaction (Fig. 7). In this type of reaction the lowest layers of cells may in places be lifted from the basement membrane and are to be found arching above the wandering cells, polymorphonuclear and mononuclear in type. This picture may persist in the trachea until the epithelial cells have developed basal bodies beneath the cuticular border in process of becoming ciliated.

Evidences of multiplication of cells in the deepest layer (corresponding to the stratum germinativum) are few as would be expected inasmuch as these cells are capable of undergoing differentiation without replacement. Mitotic figures were found in small numbers usually, however, in the later stages of repair (Figs. 5, 7 and 8).

Summarized, the sequences in the return to the normal epithelium are vacuolar degeneration of cells and leucocytic infiltration above the layer of undifferentiated cells with the formation of two strata, an upper non-viable stratum and a lower stratum, of which every cell is capable of multiplication or differentiation. The upper stratum disappears by degeneration and lysis (probably both autolysis and heterolysis are involved) and the lower stratum develops into cells normal for the location.

Where small ducts are distended by the accumulation of keratinized cells to a degree resulting in compression of the deepest cell layer, the vacuolar degeneration and separation of cells dominate the picture (Fig. 2) and the result for a time is that only a layer of flattened cells remains. These subsequently multiply and assume approximately normal cuboidal or cylindrical shapes.

We have not ascertained the time required in rats with satisfactory gain in weight for completion of epithelial repair. In several rats which resumed their normal rate of growth as shown by weight curves and less accurately by active growth of bones over periods of from 15 to 22 days following restoration of vitamin A to the diet, repair was far advanced in all situations and complete in most. In all rats where large cysts had formed, such as those frequently found in the salivary gland and particularly at the base of the tongue, the keratinizing epi-

all locations mentioned in our previous reports. We have restudied the sections of all of our A-deficient rats and guinea pigs and again conclude that the replacement of stratified keratinizing epithelium is identical in all locations and comparable in all its layers with epidermis. This restudy also permits us to state that the masking of the original epithelium is in every location a complete one. We could find no clue for identifying former normal characteristics in the replacement epithelium of the deficiency in any organ.

The phenomena of recovery are common to all locations and in each location the normal type of epithelium returns *via* differentiation of the viable cells of the lowermost layers which correspond to those of the stratum germinativum. For the duration of our experiments we must conclude that the cells capable of multiplying preserve their identities. There is, however, a suggestion that permanent loss of identity may occur after functional destruction of the organ as a whole because of the persistence of stratified keratinizing epithelium in infected cysts and in cicatrized glands for the longest repair period—43 days—of our series.

The sequences in repair indicate that in recovery from A deficiency, all cells which have progressed to a certain degree toward keratinization either degenerate *in situ* and disappear or are cast off and disappear. The line of demarcation between the viable and non-viable cells must represent that layer of cells where differentiation has progressed to an irreversible stage. This layer is close to the stratum germinativum, probably not above the second layer of cells and probably most often includes all cells above it.

The metaplasia of A deficiency and its recovery is a cycle that probably does not occur in animals in natural habitats. The fact invites speculation regarding the mechanism of repair. Repair processes following physical and chemical injurious agents have, in general, counterparts to be found in normal growth processes. The only normal process which involves mechanisms similar to those in recovery of epitheliums in A deficiency is that of the changes in the vagina of rodents during the estrous cycle. Here the sequences involved in the periodic cornification of the vaginal mucosa bear some resemblance to those of A deficiency metaplasia while the sequences concerned in the reverse changes are very similar to recovery changes in A deficiency.

phagocytosis, we give here a summary of the principal views on this subject.

Perhaps the majority would agree with Topley and Wilson's (3) description of the manner in which virulent bacteria are prepared for phagocytosis:

"Both normal opsonins and immune opsonins, or bacteriotropins, have a dual structure in a sense that they involve, or may involve, the combined action of a specific sensitizing antibody and complement; but that the very low concentration of the specific antibodies in normal serum necessitates the adjuvant action of a considerable amount of complement before its presence can be detected, so that the complementary action appears to dominate the picture, while the high concentration of the specific antibody in an immune serum reduces the adjuvant action of the complement to a mere enhancement of an effect which takes place in its absence."

There is not, however, universal agreement with this simple conception. Neufeld (4), one of the acknowledged authorities on this subject, maintains that there are two heat-stable phagocytic antibodies distinct from one another: (a) the tropin, either normal or immune, which does not require the aid of complement to bring about phagocytosis; (b) the opsonic amboceptor, either normal or immune, which does require the addition of complement to activate it. Muir (5) states that although specific opsonization (as distinct from tropinization) is always dependent on the combined action of a specific antibody and complement, nevertheless complement may unite directly with bacteria to bring about a non-specific opsonization.

Owing to the looseness with which the words "opsonin" and "tropin" have been applied to various phagocytic phenomena in the literature, we give here, to avoid confusion, the sense in which these terms have been employed in the ensuing description of our experiments: "Opsonin" is that property of unheated serum which prepares organisms for phagocytosis. "Tropin" is that property of heated serum which, by itself, prepares organisms for phagocytosis. We have used these terms in the description of our experiments because they are familiar to most workers in this field. Nevertheless, on the basis of our experimental results, in the discussion we shall urge the rejection of the term "tropin" and suggest a modification in the sense of the term "opsonin."

The experiments to be described deal primarily with the rôle of the anti-type-specific carbohydrate antibody (hereafter to be alluded to as the anticarbohydrate antibody) as an opsonic or tropic agent, and with a study of the properties of normal and immune sera which promote phagocytosis after this antibody has been eliminated by the addition of the specific carbohydrate in appropriate amounts. During the course of this work, the state of the organisms themselves has not been disregarded, and studies have been made on the phagocytosis of

that the mitochondrial apparatus was not the seat of the primary injury in A deficiency and suggested that nuclear changes were important. Obviously if there are two sorts of chromatin, one concerned with preservation of identity (idiochromatin) the other with cell function (trophochromatin), we have in the cycle of A deficiency metaplasia and recovery, a possible means of securing appropriate material for this study.

CONCLUSIONS

1. In vitamin A deficiency, the replacement stratified keratinizing epithelium is morphologically identical in all locations.
2. All cells of the lowermost layer of the replacement epithelium have proliferative power as in the stratum germinativum of epidermis.
3. In recovery, in spite of the complete morphological masking, the epithelium in each region returns to its normal type.
4. The important histological features of repair involve removal of the layers of cells irreversibly differentiated towards keratinization and direct differentiation of the stratum germinativum into the normal type. These take place simultaneously.
5. The histological sequences observed in the removal of cells above the stratum germinativum indicate that autolysis as shown by vacuolar degeneration and heterolysis as shown by leucocytic infiltration are involved.
6. The cycle of A deficiency metaplasia and recovery affords an experimental method available for the correlation of nuclear chromatin and types of cytoplasmic activities.

EXPLANATION OF PLATES

All illustrations are from camera lucida drawings at approximately $\times 700$. Modified Giemsa staining with Zenker fixation and paraffin embedding.

PLATE 36

- FIG. 1. Uterus, mid-portion. Rat 411. 170 days on deficient diet, 15 days on recovery diet. Initial weight 132 gm. Maximum weight 159 gm. At end of deficient diet, 105 gm. On the recovery diet it gained 9 gm. in 7 days; final weight not recorded. Note vacuolar degeneration, leucocytic infiltration, hyaline degeneration of superficial cell and cells of basal layer assuming columnar shapes. The line of demarcation between viable and non-viable strata shows clearly.
- FIG. 2. Submaxillary gland ducts. Rat 401. 161 days on deficient diet, 22 days on recovery diet. Initial weight 81 gm. Maximum weight 114 gm. At end of deficient diet, 87 gm. On the recovery diet it gained 30 gm. in 15 days;

contents were mixed by rapid rotation of the tube after the addition of each substance. The tubes were then sealed and warmed by immersion in water at 37°C. for a few minutes. After being dried, the tubes were put into a revolving box (rotating at a rate of 15 R.P.H.) placed in a 37°C. incubator. At the end of 30 minutes, the tubes were opened, smears of the contents were made and stained with Wright's stain. In general, the organisms in 50 polymorphonuclear neutrophils were counted, but where the number of phagocytosed cocci was few, making the result doubtful, the bacterial content of 100 cells was recorded. In counting, a diplococcus was regarded as a single organism. The accuracy of the technique which has been described was checked in this manner: 5 smears were made from each of 2 tubes containing 0.5 cc. of defibrinated blood, and 1 drop of a suspension of pneumococci, after the tubes had been rotated at 37°C. for half an hour. The organisms in 50 leucocytes were counted in each smear. The counts of the 5 smears made from the contents of the 2 tubes were: 425, 447, 460, 451, 493, and 473, 479, 511, 502, 485. The maximum deviation from the mean of the 10 counts is 10 per cent, and the maximum difference between the highest and the lowest count is 20 per cent of the latter.

The investigation of the weak tropic action of heated normal human serum demanded a modification of the technique described above. To the serum, which had been heated to 56°C. for 30 minutes, were added the various materials to be tested and then the bacterial suspension. The tubes were then placed in the ice box at 4°C. for 18 hours, after which washed corpuscles were added in equal volume. The mixtures were rotated for 30 minutes at 37°C. in the usual manner, and the phagocytic counts made. In the study of the tropic action of immune serum, the dilution of antiserum chosen was added to a mixture of heated normal serum and washed corpuscles, and after the addition of the various reagents and organisms, the tubes were incubated for 2 hours in the rotating machine. Essentially the same procedure was adopted in investigating the opsonic action of immune serum, save that a dilution of unheated normal serum was also added. Heated normal serum was used to suspend the corpuscles instead of normal saline solution, since it was found that in the latter medium the leucocytes showed little or no capacity to ingest sensitized organisms.

Bactericidal tests with defibrinated blood were carried out by the technique described by Ward in a previous communication (2).

The Opsonic Action of Normal Human Serum

The Determination of an Amount of Specific Carbohydrate Necessary to Neutralize the Homologous Antibody in Normal Serum.—As a basis for these experiments on phagocytosis, it was necessary to demonstrate the presence of the small amount of anticarbohydrate antibody in normal serum. The most delicate test we have at our disposal for detecting the presence of this antibody is the determination of the



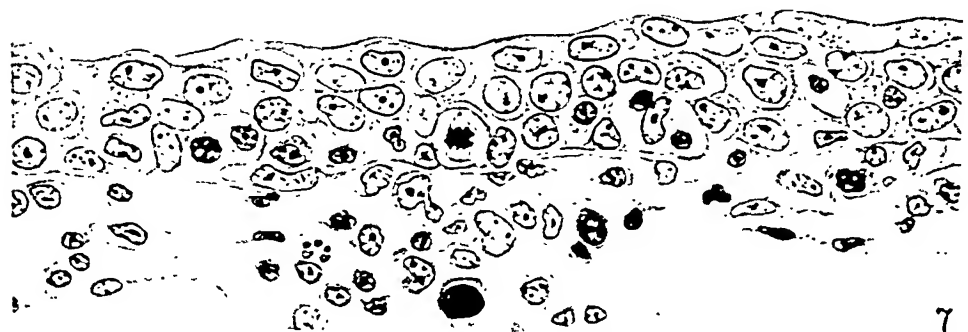
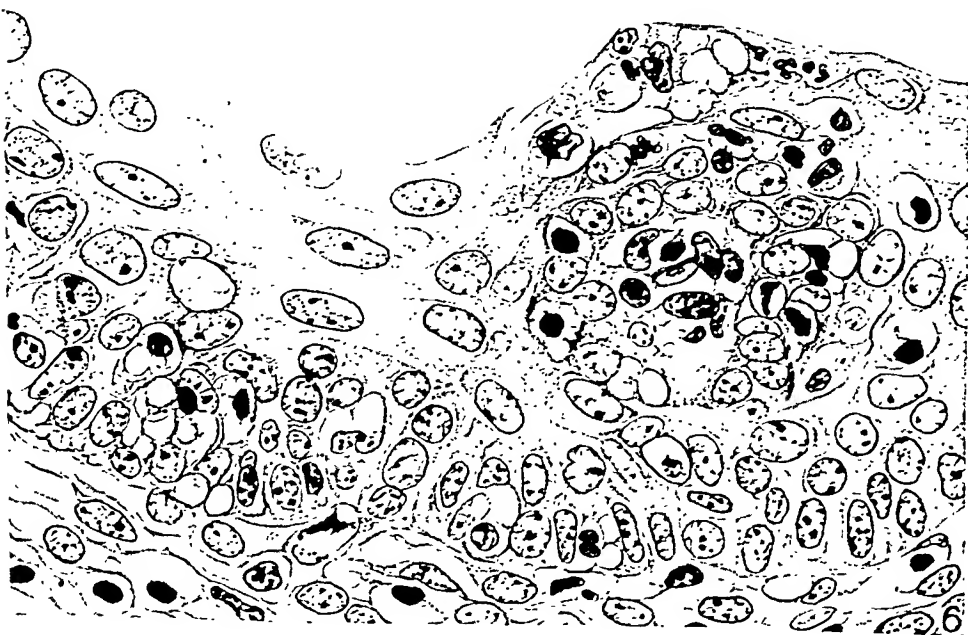
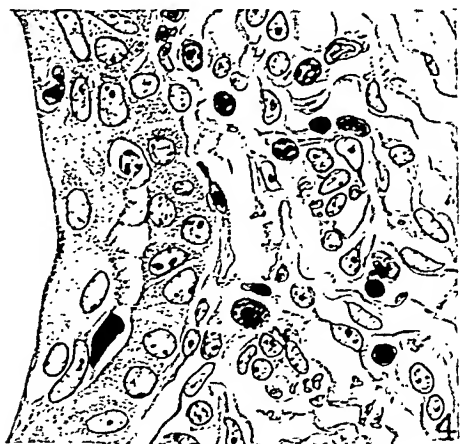
which removes the bactericidal power of normal blood, does not invariably render ineffective the phagocytic mechanism. This is strikingly shown by the results recorded in Table II. In this experiment, where 24 hour cultures were employed, it will be observed that in no case did the addition of the specific carbohydrate completely suppress the phagocytosis, and in only one case was the phagocytosis by the normal blood alone reduced more than two-thirds by the presence of the carbohydrate. The results of an investigation into this residual phagocytosis will be presented subsequently.

TABLE II

	Type of pneumococcus	Concentration of carbohydrate	No. of organisms phagocytized by 50 cells	Cells taking part
				<i>per cent</i>
Normal Blood E	I	0	512	100
	I	Type I, 1:800	249	98
	II	0	583	100
	II	Type II, 1:800	43	38
	III	0	818	100
	III	Type III, 1:800	525	100
Normal Blood W	I	0	183	96
	I	Type I, 1:800	166	92
	II	0	271	98
	II	Type II, 1:800	125	78
	III	0	617	100
	III	Type III, 1:800	630	100

Another experiment, the details of which are not given here, showed that the reduction in the degree of phagocytosis produced by the specific carbohydrate is, as one would expect, type-specific.

When, however, instead of 24 hour cultures, 8 hour cultures were used in the same experimental procedure, the specific carbohydrate exerted a much stronger antiphagocytic effect, except in the case of Type I. When the organisms were taken directly from the mouse peritoneum, the influence of the specific carbohydrate was still more marked. The experimental data of these two experiments are assembled in Table III. In addition to showing the presence of an opsonic factor distinct from the anticarbohydrate antibody in the phagocytosis

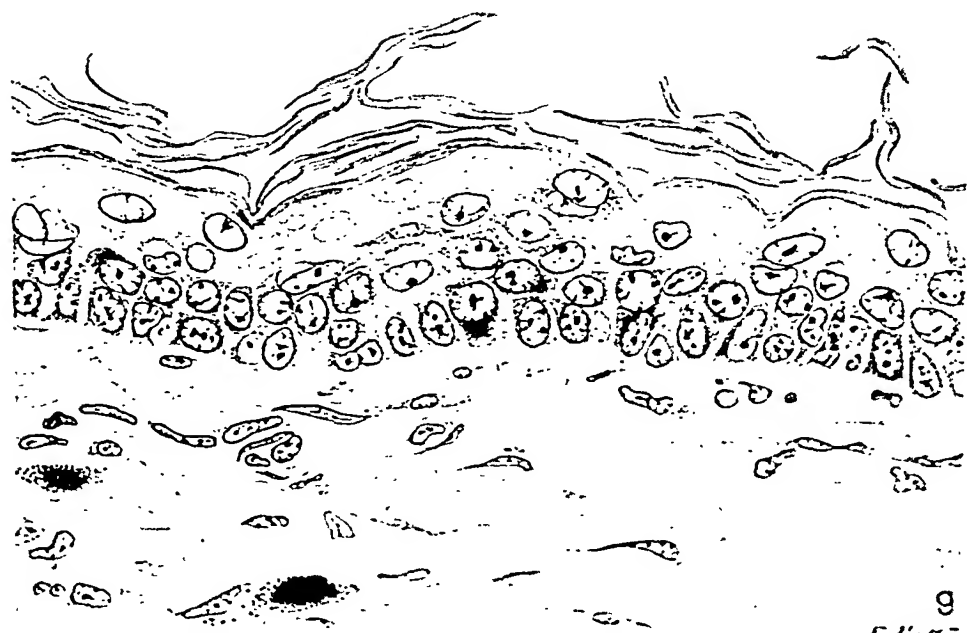
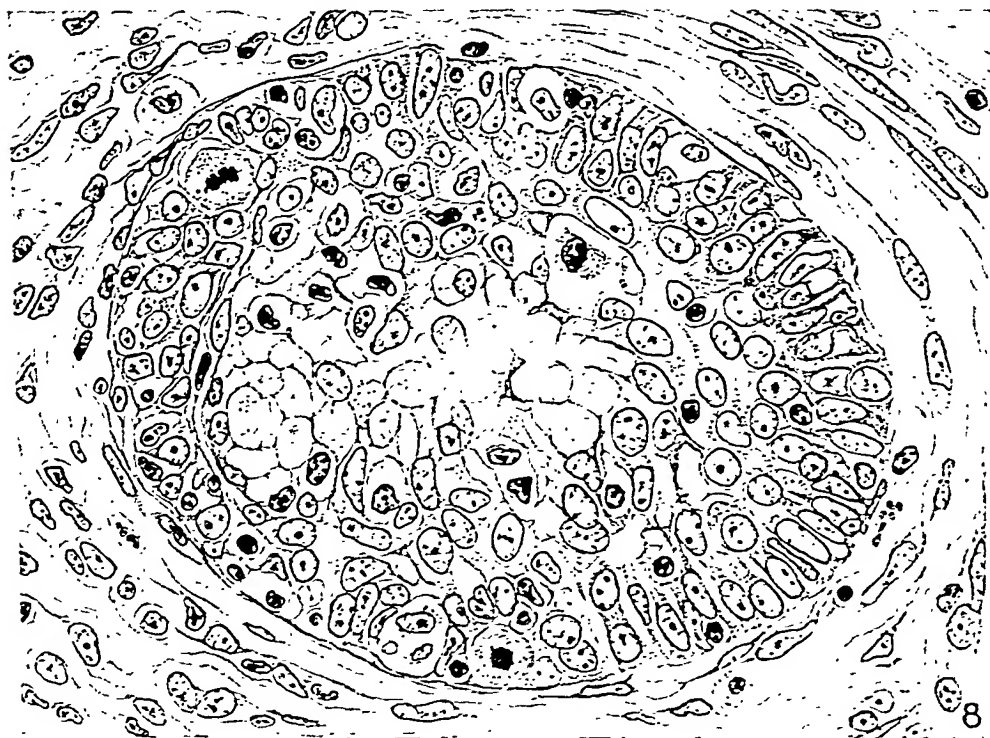


Our own work has not borne this out, but the difference in technique may well account for the discrepancy.

The Analysis of the Opsonic Mechanism in Normal Human Blood.—It remained to determine whether the anticarbohydrate and residual factors were heat-stable and in conjunction with complement brought about immediate phagocytosis according to the description of Topley and Wilson (3), to which allusion has already been made. The experimental evidence on which this is based is largely derived from the work of Dean (11) and Cowie and Chapin (12). The latter authors showed that, in the case of the *Staphylococcus albus*, an amount of unheated normal serum, in itself practically without any opsonic effect, was able to reactivate completely normal serum, the opsonic activity of which had been almost entirely removed by heating. We have attempted, unsuccessfully, to confirm their results, employing Type I and Type II pneumococci as the test organisms. Our results showed that it was impossible to reactivate the heated normal serum with diluted unheated normal serum, and therein they agree essentially with those obtained many years ago by Wright and Douglas (13), working with the staphylococcus. Perhaps the success of Cowie and Chapin's procedure lay in their choice of an organism of low virulence. As we shall see, the conclusion of Cowie and Chapin, that opsonic action depended on the combined effect of a thermostable element with complement, is correct, but, to demonstrate its validity for the virulent pneumococcus, a much greater concentration of complement was found to be necessary in order to reactivate the heated normal serum. As a source of complement which could be used in the highest possible concentration and still be free of antibody, the serum of infants below the age of 12 months was employed. The complement titre of such sera for sensitized sheep red blood cells was in general as high as that of adult sera, but the opsonizing action was negligible. In Table IV, we present the results of an experiment in which the undiluted adult Sera E and W and undiluted infant's serum were used in equal proportions. Washed corpuscles and organisms were added to these mixtures.

The conclusions to be drawn from these results are as follows:

1. The infant's serum exerted a negligible degree of opsonic action for Types I and II pneumococcus (Tubes 1 and 6). These small counts in the presence of abundant complement indicate that Muir's



adjuvant action of a thermolabile constituent, other than complement, in the adult serum.

5. The residual factor is thermostable (Tubes 3 and 8); and the differences between the counts of Tubes 2 and 3 and of Tubes 7 and 8 indicate, without proving conclusively, that the anticarbohydrate antibody is also heat-stable. Other experiments that we have performed leave no doubt that this antibody, as it occurs in normal serum, is resistant to heat.

The Nature of the Residual Factor.—The last experiment demonstrated that part of the phagocytosis of Types I and II pneumococcus by normal blood was due to a factor, other than the anticarbohydrate

TABLE V

	Type of pneumococcus	Concentration of carbohydrate	No. of organisms phagocytosed by 50 cells	Cells taking part
				<i>per cent</i>
Unabsorbed unheated normal serum + washed corpuscles	I	0	392	100
	I	Type I, 1:800	185	86
	II	0	351	100
	II	Type II, 1:800	141	86
Type I-absorbed unheated normal serum + washed corpuscles	I	0	21	18
	I	Type I, 1:800	8	8
	II	0	186	82
	II	Type II, 1:800	79	60

antibody, acting in conjunction with complement. It further made clear that this factor was resistant to a temperature of 56°C. This characteristic suggests its inclusion among the natural antibodies of the serum. Were it possible to show that it is type-specific in its action, it would strengthen the probability of its identification as an antibody. Our evidence in regard to its type specificity is not entirely conclusive. In our hands, the type-specific absorption of the opsonins of normal serum proved difficult, although Sia (14) reported successful results in this kind of experiment. We, however, observed a marked reduction of the opsonic content of the serum for a heterologous type, and this with practically no diminution in the complement content of the absorbed serum. We give in Table V the results of one of the

AN ANALYSIS OF THE OPSONIC AND TROPIC ACTION OF NORMAL AND IMMUNE SERA BASED ON EXPERI- MENTS WITH THE PNEUMOCOCCUS

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INTRODUCTION

Recent investigations (1, 2) on the pneumococcidal power of serum-leucocyte mixtures and of defibrinated human blood have demonstrated that the specific carbohydrate of the pneumococcus exerts a strong and type-specific antibactericidal action in such systems. As it is well known that ingestion of the pneumococcus by the leucocytes is essential for the destruction of this organism, the suggestion has been made by Sia (1) and Ward (2) that the specific carbohydrate inhibits the bactericidal properties of these systems by uniting with its corresponding antibody, and thus completely preventing the opsonization and subsequent phagocytosis of the organisms. For they thought it probable, in the absence of direct experimental evidence, that this antibody was an indispensable factor in the process of opsonization. We have found, however, that the addition of a quantity of the specific carbohydrate which will suppress the bactericidal action of defibrinated human blood does not prevent, under certain conditions, the phagocytosis of virulent pneumococci.

With this observation as a basis, we were led to reinvestigate the phagocytic mechanism in the case of the pneumococcus. The experimental results thus obtained not only served to clarify this special problem, but seemed to us to have a general application to the various theories of phagocytosis, in that they elucidate the function of a single well defined antibody in the mechanism of this phenomenon. Since even now there is not universal agreement concerning the nature and mode of action of the various serum constituents taking part in

has been confirmed. It is logical to think that this residual tropic factor is the same as the residual opsonic factor, and that in the heated normal serum this factor acts by itself, and in the unheated normal serum its effect is markedly enhanced by the presence of complement.

In parallel experiments in which 8 hour cultures and organisms from the mouse peritoneum were used, no tropic action could be demonstrated except that the anticarbohydrate tropin of Serum E was able to bring about phagocytosis in both instances in the case of Type II.

The Effect of the Specific Carbohydrate on the Tropic Action of Immune Serum.—The results of an experiment planned to determine the effect

TABLE VI

	Type of pneumococcus	Concentration of carbohydrate	No. of organisms phagocyted by 50 cells	Cells taking part <i>per cent</i>
Heated Serum E + washed corpuscles	I	0	81	22
	I	Type I, 1:800	17	4
	II	0	174	32
	II	Type II, 1:1,600	3	4
	III	0	451	52
	III	Type III, 1:1,600	36	44
Heated Serum W + washed corpuscles	I	0	2	4
	I	Type I, 1:800	0	0
	II	0	0	0
	II	Type II, 1:1,600	1	2
	III	0	197	50
	III	Type III, 1:1,600	53	50

of the specific carbohydrate on the tropic action of the immune serum are summarized in Table VII. Here it will be seen that the addition of the homologous carbohydrate completely abolishes the tropic action of the immune serum, except in the case of the Type I antiserum. In the latter, the failure of the Type I carbohydrate completely to nullify the phagocytosis is probably due to the comparative weakness of this carbohydrate in its neutralizing properties. It is also evident that the action of the various carbohydrates is strictly specific. With the possible exception of Type I antiserum, there is no suggestion that a factor analogous to the residual factor in the normal serum is present

cultures of varying age, and of pneumococci taken directly from the animal body.

Materials

The following materials were used in the course of these experiments.

Human blood defibrinated by shaking with beads and used on the same day it was withdrawn. In the most important experiments the blood of the authors was used, neither of whom had any history of pneumococcus infection. In certain other experiments, adult blood obtained from the Wassermann dispensary was employed. In two experiments, we made use of infants' blood, the infants being 7 and 12 months old, respectively. When washed corpuscles were required, the defibrinated blood was centrifuged, the supernatant serum removed, and the red and white corpuscles washed three times with normal saline solution. In the cases where inactivated serum was added, it was heated to 56°C. for 30 minutes.

Particular pains were taken to maintain the virulence of the three types of pneumococci employed. The organisms were passed through mice at least once a week and during most of the work, twice a week. Cultures for use in the experimental work were grown in rabbit blood infusion broth, and in order to eliminate any antiphagocytic effect of the soluble products of the pneumococci in the culture broth, the culture was centrifuged shortly before using, and the organisms suspended in sufficient fresh broth to yield a threefold concentration of the pneumococci. To obtain organisms fresh from the animal body, a mouse was injected with 0.5 cc. of a 1:200 dilution of a 24 hour broth culture. The mouse was killed after 12 hours, and the peritoneal cavity washed out with about 1.0 cc. of broth. The organisms were separated from the washings by centrifugation, and resuspended in the same amount of fresh broth. When organisms were used for absorption, they were grown in 0.1 per cent dextrose infusion broth at 37°C. for 18 hours. The quantity of serum to be absorbed was added to the organisms centrifuged from an equal quantity of the broth culture. The mixture of serum and organisms, after standing together at 37°C. for half an hour, was centrifuged at 2,000 r.p.m. for 45 minutes. The serum thus absorbed was removed from the organisms.

The specific carbohydrates were prepared according to the procedures described by Avery and Heidelberger (6, 7).

The Type I and Type II antisera used were prepared in rabbits and the Type III antiserum in a horse.

Technique

In carrying out the experiments, the following technique was employed:¹ 0.5 cc. of defibrinated blood, or a mixture of serum and washed corpuscles was placed in a pyrex glass tube 10 cm. long, 7 cm. inside diameter, and the organisms and various reagents added in this order: antiserum, specific carbohydrate, organisms. The

¹ For more complete details of this technique, see article by Ward (2).

addition of complement did not increase the amount of phagocytosis due to the antiserum, in contrast to those in which an adjuvant action of the thermolabile substance was demonstrated. However, this distinction has long somewhat confused our conception of the factors concerned in the mechanism of phagocytosis, and on the basis of the evidence presented below, is in our opinion unnecessary.

It has been shown already (Table VII) that the tropic action of a diluted Type III antiserum is caused by the Type III anticarbohydrate antibody. If it could be demonstrated that a small amount of complement increased the amount of phagocytosis, and that this increase

TABLE VIII

	Type of pneumococcus	Concentration of type-specific antiserum	Concentration of carbohydrate	Concentration of unheated normal serum	No. of organisms phagocytosed by 50 cells	Cells taking part
						<i>per cent</i>
Heated normal serum + washed corpuscles	III	1:800	0	0	182	22
	III	1:800	Type I, 1:800	0	326	40
	III	1:800	Type II, 1:800	0	367	46
	III	1:800	Type III, 1:800	0	0	0
	III	0	0	0	1	2
	III	1:800	0	1:16	740	66
	III	1:800	Type I, 1:800	1:16	1,097	96
	III	1:800	Type II, 1:800	1:16	886	80
	III	1:800	Type III, 1:800	1:16	56	34
	III	0	0	1:16	38	25

was due to the anticarbohydrate antibody acting in conjunction with complement, then Neufeld's contention, that there were two distinct antibodies involved, would be proved to be incorrect. Table VIII contains the results of an experiment in which a Type III antiserum is shown to exert a type-specific tropic action by virtue of its anticarbohydrate antibody. Here it may also be seen that this tropic action is markedly increased by the addition of a small amount of unheated normal serum. In both cases, however, when the specific carbohydrate is added, the effect of the anticarbohydrate antibody is removed, showing clearly that the same antibody can act as an immune tropin or as an immune opsonin, to employ the terminology of Neufeld.

bactericidal titre of defibrinated blood against the virulent organism (8). The same test offers the most accurate method of ascertaining the quantity of specific carbohydrate required to neutralize this antibody in normal and immune serum. In Table I, the results recorded demonstrate the effect of the specific carbohydrate on the bactericidal power of the two bloods which have been used in most of the experiments. The type specificity of the antibactericidal action of these substances is not shown, since this has been pointed out in previous papers (1, 2). From Table I it can be seen that in the case of Normal Blood E, the bactericidal power of the blood against Type I

TABLE I

	Type of pneumococcus	Concentration of carbohydrate	Maximum No. of diplococci killed by 0.5 cc. blood
Normal Blood E	I	0	70,000
	I	Type I, 1:800	7
	II	0	40,000
	II	Type II, 1:1,600	0
	III	0	4,000
	III	Type III, 1:1,600	0
Normal Blood W	I	0	0
	I	Type I, 1:800	0
	II	0	40,000
	II	Type II, 1:1,600	0
	III	0	40,000
	III	Type III, 1:1,600	0

pneumococcus is reduced 10,000 times by the addition of an amount of specific carbohydrate which was used subsequently in the course of the phagocytic experiments. In the case of Types II and III, and in that of all three types with Normal Blood W, the bactericidal titre was reduced to nil. It will also be noted that Normal Blood W, without the addition of the carbohydrate, possessed no bactericidal power against Type I, which indicated that this blood was completely lacking in Type I anticarbohydrate antibody.

The Effect of the Specific Carbohydrates on the Opsonic Action of Unheated Normal Serum.—Allusion has been made already in this paper to our observation that an amount of the specific carbohydrate

It had been determined previously that the phagocytic effect of the antiserum in the concentration chosen for the experiment was entirely removed by the addition of an appropriate amount of the specific carbohydrate. This fact clearly indicates that the phagocytic antibody involved in the experiment is the anticarbohydrate antibody.

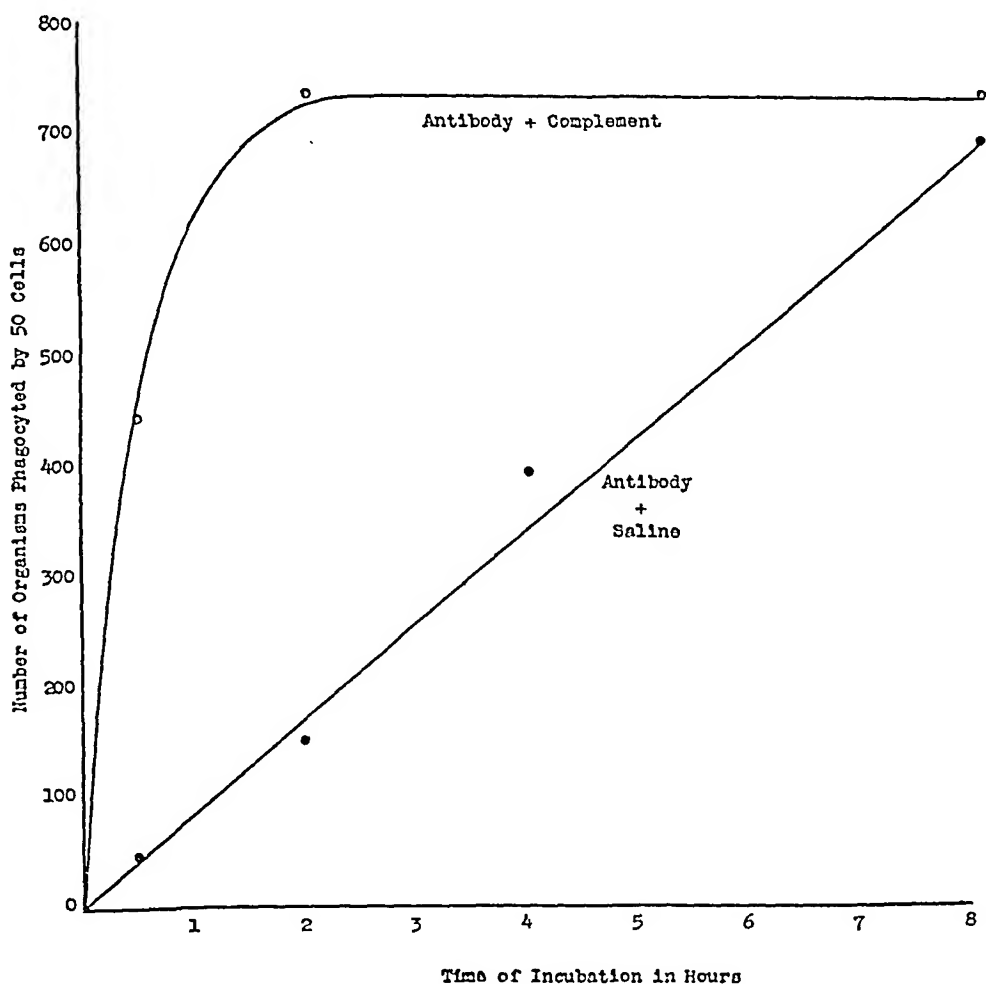


FIG. 1. Phagocytosis with and without complement.

The results obtained in the experiment are summarized in the form of a graph (Fig. 1). It will there be noted that the phagocytosis due to the antiserum alone increases at a uniform rate throughout an interval of 8 hours, but where complement is present the curve of the phagocytic count rises steeply and attains its maximum after 2 hours at the latest.

of 24 hour cultures, these experiments indicate that as the organism approaches and attains to the state in which it exists in the animal body, the less effective is the residual factor, and the more necessary is the anticarbohydrate factor in causing phagocytosis. This generalization does not apply to the Type I pneumococcus, where even the organisms taken from the mouse are susceptible to the opsonic action of the residual factor.

Our findings in respect to the Type III organisms may explain the old observations of von Gruber and Futaki (9). These authors noted

TABLE III

	Type of pneumococcus	Concentration of carbohydrate	No. of organisms from 8 hr. culture phagocyted by 50 cells	Cells taking part	No. of organisms from mouse peritoneum phagocyted by 50 cells	Cells taking part
				<i>per cent</i>		<i>per cent</i>
Normal	I	0	408	98	339	98
Blood	I	Type I, 1:800	438	96	130	72
E	II	0	544	98	73	74
	II	Type II, 1:1,600	0	0	1	2
	III	0	500	100	4	6
	III	Type III, 1:1,600	11	8	0	0
Normal	I	0	269	95	14	12
Blood	I	Type I, 1:800	235	84	18	12
W	II	0	336	82	8	12
	II	Type II, 1:1,600	6	6	10	12
	III	0	329	96	4	6
	III	Type III, 1:1,600	104	54	4	4

that, whereas fully virulent but uncapsulated anthrax organisms were readily phagocyted both *in vitro* and *in vivo*, the same strain when capsulated was completely resistant to phagocytosis. One would venture to predict that it will be found that the virulent uncapsulated anthrax bacilli can be opsonized by a serum component corresponding to the residual factor mentioned above in the case of the pneumococcus, but that this factor is powerless to bring about the phagocytosis of the capsulated organisms. Robertson and Sia (10), in their work on the opsonization of Type I pneumococci by dog's blood, found an absolute difference in the degree of phagocytosis of old and young cultures.

For if the organisms are dispersed and yet prepared for ingestion, it is obvious that the chance of contact with the leucocytes is greater than where the organisms are aggregated, as occurs when phagocytosis is delayed. This reasoning is supported by an experiment in which the organisms were allowed to agglutinate in unheated and heated serum for some hours at 4°C., and then exposed to the action of washed leucocytes. The distribution of the organisms among the phagocytes was typical in both cases of that which is found in delayed phagocytosis.

DISCUSSION

It has been clearly demonstrated in the foregoing experiments that the type-specific anticarbohydrate antibody is capable by itself of preparing the pneumococcus for phagocytosis in heated normal and immune serum,—in other words, it may act as a normal or immune tropin. With the effect apparently increased in the presence of complement, the same antibody may be responsible for the opsonic action of unheated normal and immune serum. In the case of immune serum, at least, we have presented evidence which indicates that this augmentation due to complement is rather an acceleration in the rate at which phagocytosis takes place than any absolute enhancement of the phagocytic count. For it is apparent from the experiment presented that if this antibody is allowed to act on the organism for a sufficient length of time, the degree of phagocytosis reaches the same approximate level, whether complement be present or absent. The practical difficulty of prolonged exposure has made similar experimentation with normal serum impossible, but by analogy it is reasonable to suppose that complement has no other function than that of accelerating the phagocytosis of pneumococci by unheated normal serum.

If it is permissible to generalize from the data obtained by the study of one organism, it appears that the fundamental reaction, which occurs when an organism is prepared for phagocytosis either by normal or immune serum, is the union of a specific antibody with its antigen. This is essentially the conception held by the majority of observers and expressed by Topley and Wilson (3) in the passage quoted in the introduction. Further, Mudd and his associates (18) have shown that this is the underlying principle also in the phagocytosis of colloidal particles coated with protein and subsequently treated with homologous protein antiserum. However, divergent views and experimental data not strictly in accordance with this generalization have served to

(5) hypothesis, which states that complement alone can bring about phagocytosis, is untenable. Experiments with the blood of a different infant, which was incapable of exerting any phagocytic action whatever, establish the fact that complement by itself, at least in the case of the virulent pneumococcus, is incapable of inducing phagocytosis.

TABLE IV

	Tube No.	Type of pneumococcus	Concentration of carbohydrate	No. of organisms phagocytized by 50 cells	Cells taking part
					per cent
Heated infant's serum + unheated infant's serum + washed corpuscles	1	I	0	3	5
Heated Serum E + unheated infant's serum + washed corpuscles	2	I	0	93	75
Heated Serum E + unheated infant's serum + washed corpuscles	3	I	Type I, 1:800	63	61
Unheated Serum E + heated infant's serum + washed corpuscles	4	I	0	90	69
Heated Serum E + saline + washed corpuscles	5	I	0	0	0
Heated infant's serum + unheated infant's serum + washed corpuscles	6	II	0	14	16
Heated Serum W + unheated infant's serum + washed corpuscles	7	II	0	109	77
Heated Serum W + unheated infant's serum + washed corpuscles	8	II	Type II, 1:1,600	70	60
Unheated Serum W + heated infant's serum + washed corpuscles	9	II	0	90	72
Heated Serum W + saline + washed corpuscles	10	II	0	0	0

2. The opsonic property of Sera E and W was removed by heating (Tubes 5 and 10) and then restored by the addition of the fresh infant's serum (Tubes 2 and 7).
3. That this restoration is complete is shown by the phagocytic counts when the same quantity of unheated adult serum was added to heated infant's serum (Tubes 4 and 9).
4. The restoration of the opsonic activity of the heated adult serum by the unheated infant's serum eliminates the possibility of any

medium were readily taken up by the leucocytes, whereas these organism when derived directly from the animal body were wholly resistant to phagocytosis by normal blood. In this study we have concerned ourselves rather with the mechanism of phagocytosis than with the correlation of this phenomenon with resistance to infection, but it is obvious that in any attempt to link up phagocytosis with infectious processes, more attention should be paid to the organism as it exists in the body than to the same strain growing in a test-tube.

CONCLUSIONS

1. In normal unheated human serum, virulent pneumococci may be prepared for phagocytosis by two separate antibodies, acting in conjunction with complement. One of these is the type-specific anticarbohydrate antibody reacting with the carbohydrate fraction of the pneumococcus. The other is probably also a type-specific antibody, but quite distinct from the former, and therefore must react with a different antigenic constituent of the bacterium.

2. In the normal human serum heated to 56°C., these two antibodies may, after prolonged contact with the organism, promote phagocytosis of the pneumococcus without the adjuvant action of complement.

3. Although these two antibodies are equally effective in the phagocytosis of 24 hour culture organisms by normal blood, the anticarbohydrate antibody tends to become the predominant factor as the pneumococci approach the state in which they exist in the animal body.

4. In so far as we have been able to show, the anticarbohydrate antibody is the only antibody in immune serum which can induce phagocytosis. This substance by itself is active in a phagocytic system, but just as in the normal serum, complement enhances its effect. The failure to demonstrate the presence in the immune serum of an antibody, distinct from the anticarbohydrate antibody, analogous to that found in the normal serum, may be due to the experimental difficulty of removing all the anticarbohydrate antibody from a concentrated immune serum.

5. Thus it is seen that a single well defined antibody (the anticarbohydrate antibody) may be responsible for the phagocytic action of normal unheated serum, normal heated serum, inactivated immune serum, and immune serum activated by complement. These facts

more successful absorption experiments, in which a normal serum was absorbed with Type I pneumococcus. Here, in addition to showing a type-specific reduction in the total opsonic effect (Type I from 392 of 21; Type II from 351 to 186), it will be noted that there is also a type-specific reduction in the phagocytosis, due to the residual factor (Type I from 185 to 8; Type II from 141 to 79). 4 per cent only of the residual factor remains in the case of the homologous organism, whereas 56 per cent was left for the heterologous pneumococcus. Although this is not a conclusive experiment, we believe that this opsonizing agent is type-specific. Additional evidence in support of this opinion may be found in the quantitative variation in the phagocytic activity of Bloods E and W against the three types of pneumococcus after the anticarbohydrate factor has been eliminated (Tables II and III). For if the residual factor was non-specific, the titre of the residual phagocytosis for all three types of pneumococcus would presumably be higher or lower in Blood E than it is in Blood W, but this is not the case.

The Tropic Action of Heated Normal and Immune Serum

The Effect of the Specific Carbohydrate on the Tropic Action of Heated Normal Human Serum.—So far as we are aware, there is no reference in the literature concerning the occurrence in heated normal human serum of tropins for the pneumococcus, although Robertson and Sia (10) have demonstrated that heated swine serum promotes phagocytosis of the Type I pneumococcus. Employing the technique described at the beginning of the paper, it was possible to show, as will be seen from an examination of Table VI, that some tropic action can be exerted by normal heated human serum on 24 hour cultures of pneumococci. It will be noted that a marked difference exists in the amount of normal tropins in the sera of the two individuals. Further, the addition of the specific carbohydrate brings about a significant reduction in the phagocytic count. That this reduction is unquestionably type-specific has been demonstrated in two experiments which are not given here in detail. Nevertheless, in the case of Types I and III, the specific carbohydrate, as in the case of the normal opsonins, does not completely prevent phagocytosis. In two other experiments, the presence of a factor causing this residual phagocytosis

in the immune serum in the dilutions that were used. Were it possible to test stronger concentrations of antiserum, a residual factor might be revealed, but the very great amount of carbohydrate required for neutralization of large quantities of the homologous antibody makes this impractical. It must therefore remain undetermined for the present whether the residual antibody is increased as a result of immunization.

The Enhancement of the Tropic Action of Immune Serum by the Addition of Complement.—We have already mentioned in the introduc-

TABLE VII

	Type of pneumococcus	Concentration of type-specific antiserum	Concentration of specific carbohydrate	No. of organisms phagocyted by 50 cells	Phagocytes taking part
					<i>per cent</i>
Heated normal serum + washed corpuscles	I	1:400	0	357	58
	I	1:400	Type I, 1:800	30	20
	I	1:400	Type II, 1:800	238	68
	I	1:400	Type III, 1:800	412	74
	I	0	0	4	2
	II	1:32	0	414	44
	II	1:32	Type I, 1:800	369	72
	II	1:32	Type II, 1:800	5	6
	II	1:32	Type III, 1:800	413	66
	II	0	0	12	4
	III	1:800	0	182	22
	III	1:800	Type I, 1:800	326	40
	III	1:800	Type II, 1:800	367	46
	III	1:800	Type III, 1:800	0	0
	III	0	0	1	1

tion the view held by Neufeld (4), who maintains that the tropin of immune serum is a non-complex substance, the action of which is not increased by the addition of complement, whereas any increment in the amount of phagocytosis observed on the addition of complement to immune serum is to be attributed to an immune opsonic amboceptor, which is distinct from the tropin and, like a lytic antibody, entirely inert in the absence of complement. This hypothesis of Neufeld was formulated to explain the results of certain experiments in which the

in diameter at the end of 24 hours' growth. These are somewhat drier in appearance than those of the freshly isolated spinal fluid strains and tend to remain discrete even when closely apposed. Under the binocular field microscope they appear domed with regular round margins and a smooth glistening surface (Fig. 1). Prolonged growth, up to 72 hours, gives rise to no secondary growth; the colonies become brownish in color and faintly granular, undergoing desiccation with the medium itself; subculture is now no longer possible.

Fresh Strains from Cerebrospinal Fluid. Smooth Forms.—The majority of strains freshly isolated from the cerebrospinal fluid form, after 24 hours' growth on blood agar plates, large flattened colonies which are pearly grey and lenticular with gradually shelving sides and a round and entire outline. The surface is smooth, or but slightly granular, and distinctly moist (Fig. 2). Single colonies may measure up to 4 mm. in diameter but there is a tendency for neighboring colonies to coalesce with the formation of a sheet-like growth. At the end of 36 hours some of these freshly isolated strains show colonies with a somewhat uneven surface due in large part, however, to the drying of the media and superimposed growth. At this time many of the strains have become transparent and glassy, sometimes with the formation of minute, glistening crystals on the colony surface such as have been observed by other authors (1, 2). At the same time, new and opaque, yellowish white growth appears at the margin or in the center of the colonies in the form of papillae or crescentic masses which grow over and obliterate the original growth. Transplantation from the original colonies is only rarely successful, but subculture can be made from the new or secondary growth during the first 48 hours after its appearance. Such subculture yields colonies somewhat more opaque and domed than those of the original culture but, while most are smaller and show less tendency to coalesce, there are some which, without any reference to distance from neighboring colonies, are larger and flattened, resembling those of the original culture and giving the plate a characteristic uneven growth. The uneven growth continues to appear for many subcultures, but the smaller colonies with their drier domed surfaces and their tendency to remain discrete become gradually more predominant, until finally the larger colonies disappear and all the colonies are of the small type, having become similar or closely similar to those of the stock strains. The secondary growth occurs in many strains for the first ten or even more subcultures, but then fails to appear any more. These small colonies, belonging to strains which may now be regarded as stock strains, remain viable for from 36 to 60 hours and are readily maintained on artificial media. While freshly isolated strains grow readily on primary culture and first subculture, this is no doubt due to a carrying over of some enriching substances present in the body fluids; and subsequent subcultures are often difficult or even impossible to make. Once, however, the strain has accustomed itself to artificial media, and especially when the secondary characteristics of morphology and behavior have developed, subculture onto solid media is simple, provided such contain some enriching substance and are sufficiently moist.

The Rôle of Complement in Phagocytosis

It is evident from the observation already presented concerning the absence of phagocytosis in unheated infants' serum (Table IV) that complement without antibody cannot induce phagocytosis in the case of the virulent pneumococci. What, then, is the function of complement when acting conjointly with antibody? In the foregoing experiments we had been impressed with the rapidity of the phagocytic reaction when complement was present. This fact suggested that possibly the only rôle of complement in phagocytosis was to increase the speed of the combination of the antibody with the organism. An experiment was therefore designed to test the validity of this hypothesis:

Two sets of 4 tubes were prepared, containing the following materials.

Set I

Heated normal human serum, 0.125 cc.

Washed human cells, 0.125 cc.

Type II antiserum—concentration, 1:128.

Unheated normal human serum—concentration, 1:32.

Type II pneumococci, concentrated 8 hour culture killed by heating to 60°C. for half an hour.

Set II

Identical with Set I, save that saline solution was substituted for the unheated normal human serum.

The tubes were sealed and placed in the rotating box at 37°C. One tube from each set was removed at the intervals of $\frac{1}{2}$ hour, 2 hours, 4 hours, and 8 hours. Smears were made of the contents, and the organisms in 100 cells were counted in each preparation. The opsonic effect of the heated normal serum together with the unheated normal serum was controlled by examining smears made from such mixtures at the end of 8 hours. The heated normal serum induced no phagocytosis at this time interval, while the slight phagocytosis due to the mixture of unheated and heated normal serum was deducted from the count of the corresponding mixture containing antiserum. The same control at 4 hours showed negligible phagocytosis.

An important detail in this experiment is the concentration of antiserum that is chosen. If the concentration is too strong, the phagocytosis due to the antiserum alone will be rapid enough to mask to a great extent the enhancing effect of the complement; if too weak, the incubation would have to be prolonged indefinitely to demonstrate the full effect of the antiserum acting by itself.

instability when subcultured on the usual artificial media (blood agar plates, serum dextrose slants and semisolid ascitic fluid agar stab). Just as the freshly isolated smooth variants tend to pass over gradually into what may be termed the stock variant, so also the rough variants, in spite of careful picking and transfer of single colonies, show an ever present tendency to pass over into the stock variant. Certain media, as 5 per cent rabbit serum peptone water or 5 per cent glycerin egg slants (6), maintain these rough variants for a while, but it is wise to make careful selection every few weeks of rough colonies plated out on 10 per cent chocolate rabbit's blood hormone agar in order to keep pure and characteristic rough types.

Other features common to the rough variants are some degree of instability and difficulty of even emulsion in normal saline; broad agglutination in phosphate buffer solutions at varying pH; and widely unspecific agglutination in serum, each monovalent type serum showing an equal or nearly equal agglutinative titre.

Mucoid Forms.—A few spinal fluid strains have been encountered which, while presenting colonies on primary isolation of normal or slightly moister appearance, develop on subculture mucoid colonies adhering firmly to the surface of solid media and tending to form glutinous strings when suspended in saline, in place of the usual homogeneous suspension. All such strains isolated to the present time, save one, have been of Type II. The factors underlying this mucoid character are as yet undetermined and apparently have little to do with the salt content of the media used, but such strains appear to be less satisfactory antigens for the production of monovalent serum than normal ones.

Fresh Carrier Strains from the Nasopharynx of Healthy Persons.—While the foregoing description includes all the more usual forms observed in freshly isolated spinal fluid strains, it is not applicable to the majority of strains recovered from the nasopharynx, of normal individuals at least. Thus, of fourteen strains obtained from the throats of normal healthy individuals with no history of meningococcal infection, only two, both typical Type II strains, have resembled the smooth spinal fluid strains on primary isolation. The others have presented colonies which resemble rather the stock strains. Thus, they are often somewhat more opaque and white; small and of uniform size; slightly domed and rather dry; and tend to remain discrete. Some authors have refused to admit these atypical nasopharyngeal strains to the group of true meningococci (7, 8), while others insist that such strains must be regarded as true meningococci in spite of atypical agglutination reactions (9, 10). The whole question is discussed elsewhere (11). Here it need only be pointed out that the atypical strains have as much, if not more claim to be regarded as true meningococci as have atypical strains of pneumococci to belong to that group.

Staining Reactions

All the strains of meningococci examined have been characteristically Gram-negative. Freshly isolated strains when emulsified in saline

Thus at the end of $\frac{1}{2}$ hour, the phagocytic effect of the antiserum is increased ninefold by the addition of complement, five times at an interval of 2 hours, twice at 4 hours, whereas at 8 hours the counts are approximately the same, whether complement be present or absent. From these results it would appear that, if the reaction is allowed to continue for a sufficient period of time, a given quantity of antibody promotes the same amount of phagocytosis, whether or not complement takes part in the process.

Complement, then, cannot increase the absolute capacity of a given concentration of antiserum to promote phagocytosis, but merely appears to do so by accelerating the velocity with which the organism is prepared for ingestion by the cells. The acceleration is probably caused by an increase in the rate at which the antibody enters into effective combination with the antigen under the influence of complement. This conception is supported by the experiments of Wright and Douglas (15), Bulloch and Atkin (16), and Sellards (17), which showed that if organisms were exposed to the action of unheated normal serum and subsequently heated to 60°C., they were phagocytosed almost as readily as organisms subjected simply to the action of the unheated serum. In contrast, as is well known, organisms which are placed in normal serum which has previously been heated to 60°C., are not taken up by the phagocytes. The probable explanation of these results seems to us to lie in the rapid union of the available antibody with the organism under the influence of complement. When once this union has taken place, the subsequent destruction of the complement has no effect on the phagocytic process.

Another effect which we have observed repeatedly and which seems to be dependent on the accelerating action of the complement is seen in the proportion of cells taking part in phagocytosis. In the unheated serum, between 90 and 100 per cent of the phagocytes usually take part. On the other hand, in heated serum the same number of phagocytosed organisms is distributed among a much smaller percentage of cells. For example, it was found in one experiment that after 2 hours' incubation, 50 polymorphonuclear leucocytes contained 182 organisms distributed among 45 out of the 50 cells when complement was present; whereas in the absence of this substance 151 organisms were distributed among 18 of the 50 leucocytes counted. It is probable that the reason for this difference lies in the fact that organisms in heated serum are agglutinated before they are ingested, unlike organisms in unheated serum, which are phagocytosed before agglutination can take place.

rule scanty, diffuse and granular growth when planted in small amounts of broth and in larger amounts grow poorly if at all, since the organisms are apparently readily "drowned." Rough variants grown in 5 per cent rabbit's serum peptone water have a diffuse granular growth.

Acid Agglutination

It has not proved possible to obtain the same clear-cut results with the acid agglutination range of meningococci as with that of many other organisms (13, 14). Nevertheless, it has been possible to show differences between fresh, stock and rough strains.

While, as pointed out above, the rough variants form unstable suspensions in normal saline, *i.e.* are "salt-sensitive," freshly isolated spinal fluid strains containing no rough variants emulsify readily and form smooth suspensions in normal saline at the usual pH of 5.5 to 6.0. Nasopharyngeal strains and stock strains usually behave in the same manner as the spinal fluid strains, but on occasion form unstable suspensions. These peculiarities are closely bound up with the acid agglutination ranges of the strains in question.

A suspension of approximately 2,000,000,000 organisms per cc. is prepared. The organisms are emulsified directly from a blood agar plate at the end of 18 hours' growth. They are not washed since the tendency to spontaneous agglutination is found to be markedly increased by washing. 0.5 cc. is mixed with an equal amount of each of ten samples of sodium phosphate buffer solution ranging in pH from 2.15 to 6.3. A control tube of suspension and normal saline is added. Certain precautions must be adopted with the saline used for the suspension and the control. Owing to the absorption of CO₂ from the atmosphere, salt solution prepared from ordinary distilled water may have a pH of 6.0 or even 5.5. Such a pH is within the acid agglutination range of many strains and will cause agglutination in the control tube. In most instances the report of salt sensitiveness in a strain is due to this factor. It may be overcome if distilled water which has been triply distilled from glass is used, and the saline is boiled shortly beforehand to expel the CO₂. Many rough strains, however, have an acid agglutination range extending beyond the pH of any unbuffered saline and such are of necessity salt-sensitive. Buffer solution and suspension of organisms are thoroughly mixed together by agitation and the tubes placed in the water bath at 56°C. for 3 hours. They are then removed and those tubes in which agglutination has occurred are noted.

By this method it has been determined that freshly isolated strains show a narrow agglutinative zone usually between pH 3.0 and pH 5.0.

set apart the subject of phagocytosis as something which is still somewhat mysterious and obscure. The demonstration that the anti-carbohydrate antibody can be the one essential serum factor in all the phenomena of phagocytosis definitely relates phagocytosis to the other serum reactions, since it is known that this antibody can be responsible for agglutination, precipitation, fixation of complement, anaphylaxis, and at least is an essential factor in the intracellular digestion and death of the pneumococcus in the blood of resistant animals. It may be pointed out in passing that this universal participation of the anti-carbohydrate antibody in all the serum reactions strongly supports the unitarian conception of antibodies advanced by Dean (19) and Zinsser (20).

It is true that in normal human serum we have shown that there is another heat-stable substance which under certain conditions can bring about phagocytosis in the absence of the anticarbohydrate antibody, but like the latter, this substance exerts a tropic and opsonic action, and therefore is presumably a separate and distinct antibody reacting with a separate and distinct antigen in the pneumococcus. Attention should be drawn here to the fact that this phagocytic factor by itself is incapable of leading to the destruction of pneumococci in a bactericidal system. This fact shows that the phagocytic capacity of a normal blood may give no indication whatever of the resistance of the animal to infection, at least as far as this resistance is correlated with the bactericidal power of the blood.

Some, at least, of the obscurity that still hangs over this subject can be laid at the door of the terminology. In our opinion, the word "tropin" should be discarded as both confusing and unnecessary. "Opsonin," however, adequately expresses the idea of preparing an organism for phagocytosis, and we would propose that this term be reserved for the antibody in the serum—whether normal or immune, heated or unheated—which unites with the organism and leads to its phagocytosis. At the same time, it would be understood that the rôle of complement in apparently increasing the degree of phagocytosis is that of a catalyst accelerating the rate of the reaction.

In concluding our discussion, we wish to emphasize the importance of the state of the organism in the phagocytic process. As an example, it has been found that Type III pneumococci growing in culture

influenzae, and a subsequent review of the literature has revealed papers by Heist and the Solis-Cohens (17) and by Matsunami (18), dealing with the action of specimens of human or rabbit blood, or of different parts of one blood on a single strain or on different strains of meningococci cultured therewith. These authors suggest (16, 17) that the ability of a strain to grow out in defibrinated blood is associated with its virulence. A virulent strain, being better able to withstand the normal defense mechanism of the body, can presumably resist and overcome the phagocytes still present and active in freshly drawn defibrinated blood, and hence will survive and grow in the blood better and in higher dilutions than an avirulent strain. The greatest dilution at which subculture can be obtained would thus be taken to yield a relative indication of the virulence of the strain.

The following method has been adopted in this laboratory to investigate fresh and stock strains. A suspension of meningococci is prepared in normal saline containing 2,000,000,000 organisms per cc. The strains are investigated as soon after isolation as possible and cultures on blood agar plates not older than 18 hours are used in preparing the suspension. Neglect of these factors will result in the use of strains and cultures which have lost some of their vitality. 1 cc. of the standard suspension is added to 9 cc. of normal saline, giving a suspension of 200,000,000 organisms per cc., and the dilution process is repeated to give regular dilutions down to 200 organisms per cc. Eight small sterile tubes are prepared, each containing 0.9 cc. of defibrinated rabbit's blood; and 0.1 cc. of the eight suspensions of meningococci is added to tubes numbered from 1 to 8. A control tube of defibrinated blood alone, No. 9, is included. Thus No. 1 contains 200,000,000 organisms and Tube 8, 20 organisms each in 1 cc. of diluted defibrinated blood. All manipulations are carried out as rapidly as possible and with precautions for sterility. The tubes are now corked and sealed with wax, and placed on a slow rotator for 15 hours at 37°C. At the end of incubation, the tubes are examined and a loopful of each is planted on a blood agar plate. Growth on the plate is recorded at the end of 24 hours, under an increased CO₂ tension of about 10 per cent. 2,000,000,000 organisms per cc. is taken as standard; therefore, Tube 1 is 10⁻¹ and Tube 8 is 10⁻⁸. If only the first tube shows growth, the strain is designated as 10⁻¹; if the first three show growth, as 10⁻³, and so on. As a rule, designation of viability of a strain can be made as soon as the tubes are removed from the rotator, for it has been noted that when growth has occurred the defibrinated blood is dark brown in color, owing apparently to methemoglobin formation. The designation obtained by this means is usually but not invariably the same as that obtained by growth on the blood agar plate.

appear to us to invalidate Neufeld's division of the phagocytic antibodies into (a) bacteriotropins (antibodies, the phagocytic titre of which is not raised by the addition of complement); (b) opsonic antibodies (antibodies, comparable to the lysins, which are only active in the presence of complement).

6. Complement alone is incapable of inducing phagocytosis of the pneumococcus. In the phagocytic process, it appears simply to increase the speed at which the reaction takes place. Its rôle may be compared to that of a catalyst in a chemical reaction.

7. On the basis of these findings, it is proposed that the term "tropin" be discarded as misleading and unnecessary, and that the term "opsonin" be retained to denote any heat-stable antibody which prepares bacteria for phagocytosis. Contrary to current usage, it would not suggest a combination of antibody with complement.

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SUMMARY

Freshly isolated strains of meningococci present a number of characteristics which can be shown to differ not inconsiderably from those of stock strains long maintained on artificial media. Rough variants of the different types can be demonstrated, either arising spontaneously *in vivo* or *in vitro*, or evoked in the laboratory by the method described by Enders. Neither the freshly isolated strains—which are smooth—nor, in most cases, the rough variants of them are stable, both showing a tendency to pass over into the stock form or variant. The stock strains in the course of transformation from the freshly isolated strains show changes in morphology and cultural characteristics, and in viability in defibrinated blood.

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EXPLANATION OF PLATE 39

FIG. 1. Stock strain of *N. intracellularis*, 18 hour growth on blood agar plate.
 × 4.

STUDIES ON MENINGOCOCCUS INFECTION

I. BIOLOGICAL PROPERTIES OF "FRESH" AND "STOCK" STRAINS OF THE MENINGOCOCCUS

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(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATE 39

(Received for publication, November 10, 1932)

A study has been made of stock and fresh strains of meningococcus obtained from various sources.¹ The stock strains, isolated in the past from cases of meningococcal meningitis, have been grown on artificial media over a period of years, while the fresh strains have been recently isolated from the cerebrospinal fluid of patients or from the throats of carriers. This paper deals with certain differences in morphology and biological characteristics in these two groups. Differences in serological reactions, in antigenicity and in antigenic complexity will be discussed in subsequent papers.

Cultural Characteristics on Blood Agar Plates

Stock or Laboratory Strains.—Strains of meningococci, whatever their type, having been grown on artificial media over a period of years, show certain morphological characteristics by which they can be recognized. Stock strains, lightly sown on blood agar plates, present small grey or whitish colonies little over 1 mm.

¹ For the stock strains, the author is indebted to the New York and the Massachusetts state boards of health, the National Institute of Health, Washington, and Dr. John Enders of Harvard Medical School. Certain of the fresh strains have been isolated from the throats of patients or normal individuals in The Rockefeller Institute. Others have been received through the kindness of the New York State Department of Health, the Departments of Medicine and Pediatrics of the Johns Hopkins and the Yale Medical Schools, the Willard Parker Hospital, New York, the National Institute of Health, Dr. O. D. Chapman of Syracuse, Mr. Eccles of the Harlem Hospital, New York, Professor R. McIntosh of the Babies Hospital, New York, Dr. G. M. Mackenzie of Cooperstown, Dr. John Norton of Detroit, and Dr. L. G. Zervas of Indianapolis.

Rough Variants.—While the above description holds true for the majority of spinal fluid strains, notable exceptions have been encountered. Enders (3) has described a method for the development of rough variants from freshly isolated strains of meningococci. All his variants have been obtained from fresh Type II strains, no success being met with Type I or stock strains. The development of rough variants has been confirmed in this laboratory and extended to fresh Type I strains. Even before the appearance of Enders' paper, however, two stock strains were discovered, one of Type IV and the other a member of the group isolated by Branham and called by her *Neisseria flavescens* (4), which showed rough and smooth variants growing side by side in the same culture (5). More recently five spinal fluid strains have been encountered which agglutinated in all four monovalent sera; and all showed the presence of rough variants. Three of these strains proved on absorption to belong to Type II, one to Type I, while the fifth remained indeterminate. In four strains rough variants were obtained which, though less granular than those obtained *in vitro*, nevertheless differed in morphology and other respects from the smooth strains. In the Type I strain a typical rough variant was found. In some cases these variants formed the greater percentage of colonies on the plate; in others they were in the minority.

These rough strains derived from various sources, while not identical in morphology, show nevertheless many essential resemblances. The colonies present a surface which is, in varying degree, roughened, and a margin which is irregular in outline; they are drier than the smooth variants. The rough variants discovered in the stock strains are the most granular of all variants yet studied. They have a surface often so wrinkled as to resemble that of a typical colony of *Neisseria sicca* (Figs. 3 and 4) and in keeping with this they emulsify with difficulty in saline and exhibit spontaneous agglutination even when the greatest care is taken in adjusting the pH. The rough colonies developed *in vitro* from Type II strains appear on first dissociation at the end of 24 hours' growth as very minute, colorless, discoid colonies, less than 1 mm. in diameter, with a flat, granular surface and an irregular margin (Fig. 5). At the end of 48 hours they are larger, up to 2 or 3 mm., with a raised, even more roughened surface and great irregularity of outline. Repeated subculture from these variants gives colonies which are larger, 2 or 3 mm. in diameter, at the end of 24 hours, but otherwise present all the appearances of the primary rough variant (Fig. 6). The variants obtained from Type I differ somewhat from those of the Type II strains. The minute discoid form has not been seen. The colonies in one instance show smooth and rounded margins but a plateau shape, with a finely granular surface and numerous deep pits. In other instances they resemble the larger colonies of the Type II R variant. The rough variants discovered thus far in spinal fluid strains have all been of the general type described for the later subcultures of Type II *in vitro* variants. It is to be noted that three out of five of these spontaneously occurring variants have been in Type II strains.

Among the features which rough variants have in common is their relative

and smeared on a slide appear as small groups, diploid forms or single organisms. Using a slight modification of the method of capsule demonstration described by Baker (12), it has been possible to show a clear zone or "capsule" around organisms of the freshly isolated strains; in stock strains this "capsule" is narrower and rough variants show no such structure. It has not been possible, however, with any of the methods used to demonstrate the presence of a true capsule comparable to that of the pneumococcus.² While with the Gram stain the organisms of the stock strains are of the same size or but slightly smaller than those of the fresh strains, the rough variants are considerably larger and show a tendency to be in groups of four (tetrads) or more organisms. These facts concerning the rough variants have been noted by Enders (3). It would seem probable that the larger forms (not the giant forms) sometimes seen in the smears of original spinal fluid cultures represent rough variants. Such, in colony form, are apt to be overlooked on the plate and are probably soon lost as a result of their instability on ordinary culture media.

Growth in Broth

Another feature serving to differentiate fresh and stock strains is their behavior in broth. It is generally believed that it is difficult to obtain plentiful growth of meningococci in any fluid medium. While this is in large part true of the stock strains, fresh strains will grow readily in Huntoon's hormone broth even without gelatin or dextrose. Growth is in the form of a heavy pellicle on the surface of the broth which breaks up when disturbed, sinking to the bottom of the flask to be replaced by a new pellicle on the surface. This form of growth occurs not only in small amounts of broth in a tube, but also in large amounts; *i.e.*, 900 cc. in a flask. Stock strains, however, show as a

² To this extent the claim to have demonstrated a capsule, made in a previous paper (5), must be modified. The "capsule" or clear zone which can be demonstrated readily by Baker's India ink method would appear to represent some difference between fresh, stock and rough organisms in or around the bacterial cell. However, it has not been possible to stain any structure outside the bacterial cell, using the usual methods of capsule staining, and it seems certain that the organism does not possess a capsule of the same kind as that found on the pneumococcus or *H. influenzae*.

isolated strains are grown on blood agar plates and used as soon as good growth appears—that is to say in 15 to 20 hours—being washed off in normal saline and adjusted to the required dilution with the help of a Gates turbidometer. After the first series of injections, the animal is allowed to rest for 5 to 7 days and the procedure is then repeated using larger doses. A third series of injections may be given, though the titre is often satisfactory after the first two. The final dose is approximately 10,000,000,000 organisms. Serum obtained in this way has a satisfactory agglutinin titre but has few if any precipitins for the soluble specific substance. Precipitins may be evoked by allowing animals to rest for 4 weeks rather than a few days between injection series, but this method of precipitin production is not as good as the one given below. A trial bleeding is made 5 days after the end of the last series of injections and if the titre proves sufficient the animal is bled immediately up to 50 or 60 cc. With care the animal may be saved and bled again after a few injections at a later date.

While the above method has proved satisfactory in the preparation of agglutinating sera, the precipitin content is usually low. A statement by Murray (1) that an interval of 6 weeks in the course of vaccination appeared to be essential for the production of sera with a high precipitin titre was tested and the following method evolved.

The animals are injected daily for 1 week with increasing doses of organisms from an initial dose of 1,000,000,000 organisms. They are then allowed to rest for a week when the procedure is repeated with larger doses; and again a third time. 5 days after this a trial bleeding can be made, but while the agglutinin titre may be high, the precipitin titre, except in rare instances, is negligible. The animals now rest for 6 weeks when the vaccination is begun anew and two series are given. Trial bleeding now shows, as a rule, a serum with a sufficiently high precipitin titre and the animal is immediately bled. If the titre is not satisfactory, a third series of injections may raise it to the required height. Besides living organisms, both formalinized and iodized antigens have been used and give even better results.

In preparing the formalinized antigen the organisms are washed off a blood agar plate with 10 cc. of a 0.2 per cent solution of formalin. The suspension so obtained stands in the ice box overnight before it is used. In the case of the iodized antigen the organisms are washed off in 10 cc. of normal saline. To this suspension is added sufficient iodine solution to render it permanently brown (about 7 or 8 cc.). The iodine solution used consists of 1.27 gm. of iodine and 5 gm. of potassium iodide in 1,000 cc. of distilled water. When this solution is added in small amounts to the suspension of organisms, the initial brown color can be seen to fade gradually (or faster if the suspension is agitated). It appears that the iodine is removed from solution possibly by adsorption to or actual chemical combination with the bacteria.

Using these treated antigens, series of injections have been given corresponding as to time intervals and quantities with those used for the suspensions of living organisms.

All agglutinations have been carried out in water baths regulated to the required

Repeated subculture broadens this zone, at first largely on the acid side, and the stock cultures show a considerably broader zone which, while quite irregular, tends to lie between pH 2.1 and pH 5.6. Rough strains usually show some agglutination over the whole range (pH 2.1 to pH 6.3) and agglutination is complete in most of the tubes.

These results are of particular interest in view of those reported by Gibbard (15) in connection with electrophoresis of meningococci. He was able to show a difference in potential difference or charge carried on the bacterial cell between fresh and stock strains. The P.D. was higher in the fresh strains, being from 36.0 to 42.0; it fell off rapidly with subculture; and stock strains showed a P.D. between 25.6 and 30.5. The range of variability was greater in stock than in fresh strains, and the P.D. tended to be greater in Types I and III than in II and IV.

Behavior in Defibrinated Blood

It has been noted above that attempts to obtain rough variants from stock strains and from most Type I strains have been unsuccessful. Rough variants are obtained after the method of Enders (3), which consists, in brief, of repeated subculture of freshly isolated strains of the organisms in defibrinated rabbit's blood, together with daily plating and selection. The strain is planted in about 0.5 cc. of defibrinated blood and slowly revolved at 37°C. for 15 hours. At the end of this time the tube is removed, a loopful plated on chocolate hormone agar and 0.1 cc. transplanted to a fresh tube of defibrinated blood with which the process is repeated. The plates are examined at the end of 24 hours' growth, preferably under a slightly increased CO₂ tension (about 10 per cent), for the presence of rough variants. In some strains these appear immediately, in others not until 20 or 25 successive subcultures have been made. Even if rough variants do not appear, the strain loses the characteristics of a fresh strain and takes on those of the stock type. Now the difficulty in obtaining rough strains from stock strains lies in the fact that the latter die out after the first two or three subcultures; *i.e.*, before the rough variants develop. This observation led to the idea that growth in defibrinated rabbit's blood might offer an additional method of differentiating stock and fresh strains. During the investigation of this point, a paper appeared by Wright and Ward (16), in which such a method was adopted with strains of *H.*

nation is much less than with the fresh strains, and, moreover, that the Type II strain shows a certain amount of cross-agglutination.

The anti-S sera used at 56°C. for 24 hours prove well nigh useless with both fresh and stock strains, owing to the very marked cross-agglutination which occurs. This cross-agglutination is evident even after 3 or 4 hours, though less than at the end of 24 hours.

Standard sera at 37°C. prove of little value. The titre of agglutination in both fresh and stock strains, especially the former, is insignificant and the results are obscured by cross-agglutination.

TABLE I

Strain	Anti-S monovalent serum															Remarks
	Type I					Type II					Type III					
	1/10	1/25	1/50	1/75	1/100	1/10	1/25	1/50	1/75	1/100	1/10	1/25	1/50	1/75	1/100	
Fresh Type I No. 17.....	2	2	3	3	4	0	0	0	0	0	±	2	3	3	4	Agglutination at 37°C. for 2 hrs. and ice box over- night
Fresh Type II No. 31.....	±	0	0	0	0	4	4	3	3	0	0	0	0	0	0	
Fresh Type III No. 7.....	3	3	3	2	0	0	0	0	0	0	3	3	2	±	0	
Stock Type I No. 123.....	2	2	1	1	±	0	0	0	0	0	1	1	1	1	1	
Stock Type II No. 383.....	±	±	0	0	0	±	±	±	2	2	0	±	±	±	±	
Stock Type III No. 57.....	1	1	1	1	2	0	0	0	0	0	1	±	1	1	1	
Fresh Type I No. 17.....	4	4	3	3	3	1	2	1	1	1	3	4	3	3	3	Agglutination at 56°C. for 24 hrs.
Fresh Type II No. 31.....	1	1	1	1	±	2	1	1	±	±	1	±	±	±	±	
Fresh Type III No. 7.....	3	3	2	2	2	3	3	2	2	2	2	2	2	2	3	
Stock Type I No. 123.....	2	2	2	2	2	2	1	1	±	±	1	2	2	3	3	
Stock Type II No. 383.....	1	±	±	±	0	2	2	2	2	2	2	2	1	±	±	
Stock Type III No. 57.....	3	2	2	2	2	2	2	1	1	1	2	2	3	3	3	

When the standard sera are used at 56°C., the results are better than at lower temperatures. Cross-agglutination, however, is present in all the lower dilutions, and it is only at 1/200 or above that the results can be interpreted. Since many freshly isolated strains and even occasional stock strains fail to agglutinate in these higher dilutions, inconclusive results are not infrequent with this technique. The method, moreover, calls for the use of a series of tubes twice as large as that used with the anti-S serum and has the disadvantage of

Using this method, fresh spinal fluid and nasopharyngeal strains, stock strains and rough variants have been examined. The results are tabulated in Table I. From this it can be seen that quite distinct differences are apparent in the several groups. Spinal strains show the highest viability. They may be divided into three groups: (1) those with high viability; (2) those with medium viability; and (3) those with low viability. When this is done, it is immediately apparent that all strains in Group 1 are of Type II; Group 2 contains the Type

TABLE I

Spinal fluid strains			Carrier strains			Stock strains			Rough strains		
Strain	Type	Virulence or viability	Strain	Type	Virulence or viability	Strain	Type	Virulence or viability	Strain	Type	Virulence or viability
439	II	10^{-7}	438	V	10^{-3}	386	I-III	10^{-3}	432/25	II	10^{-2}
442	II	10^{-6}	452	V	10^{-2}	403	I-III	10^{-1}	18 ^R	II	10^{-2}
471	II	10^{-7}	456	VI	None	383	II	None	435 ^R	II	10^{-2}
477	II	10^{-8}	453	?	None	392	II	None	434 ^R	II	None
445	I-III	10^{-4}	427	V	10^{-2}				438 ^R	V	10^{-1}
447	I-III	10^{-4}	433	V	10^{-2}				B4 ^R	IV	10^{-2}
448	I-III	10^{-4}	458	I-III*	None				157 ^R	<i>N. flavescens</i>	None
449	I-III	10^{-3}	15	II	10^{-6}						
454	I-III	10^{-2}	441	II*	10^{-2}						
460	I-III	10^{-3}	450	?	None						
436	II*	None	18	II*	None						
440	II*	None	443	II*	None						
446	II*	10^{-3}	459	II*	10^{-1}						
			422	VII	None						
			444	VII	None						

* Atypical.

I-III strains; while the strains of low viability either agglutinate broadly in all sera or contain rough variants and are indeed not typical fresh spinal strains. Of the carrier strains, only one showed a high viability and this, Strain 15, as has been pointed out above, was morphologically and culturally a typical spinal strain of Type II, which renders its high viability not surprising. The remainder of the carrier strains are of low viability. The stock strains, though giving characteristic agglutinations of Types II or I-III, have uniformly low viability, as have the rough variants.

demanding a longer period of time before the final readings can be made.

The results given in the tables are those of a single experiment. Repeated observations in the course of routine examination of freshly isolated or stock strains have served to confirm them. The fresh strains used in this experiment were of known antigenicity and necessarily not of as recent isolation as those subsequently tested in the general routine. It has been the experience in this laboratory that the fresher strains give even better agglutinations with the anti-S sera at 37°C. than those shown in Table I. Nearly all of the really fresh strains can be confidently assigned to their type on removal from the water bath at the end of 2 hours, obviating the delay of the overnight period in the ice box. While some fresh strains have been encountered in which cross-agglutination occurs, it has been found that in all cases in which this was great enough to obscure the results, it has been possible to demonstrate rough variants in the cultures.

DISCUSSION

In comparing the method of agglutination here described with those more usually adopted, certain points call for emphasis. These have to do principally with the serum used and the conditions under which the reaction is carried out.

In connection with the serum, there are two main features. The first is the choice of strains for use in serum production, and the second is the titre of agglutinins which it is considered necessary that the serum should finally attain. It is the accepted view that, in the preparation of monovalent sera, stock strains, being of known antigenicity, are as good as, indeed better than, freshly isolated strains of which the antigenic properties and complexity are problematical. It therefore becomes necessary to inquire if the freshly isolated strains have the same antigenic complex and can give rise to sera as satisfactory as those obtained by the use of stock strains. Kirkbride and Cohen (2) believe that recently isolated strains of Type I and Type III are not superior antigenically to the 13 year old stock strains used in routine serum production, while fresh Type II strains are but little better. Many observers are agreed, on the other hand, that the antigenic complex of many organisms, including meningococci (3, 5-7) does

FIG. 2. Fresh strain of *N. intracellularis*, 18 hour growth on blood agar plate. $\times 4$.

FIG. 3. Rough colony of *N. flavescens* (Branham) obtained by punctate inoculation of a blood agar plate. $\times 9\frac{1}{2}$.

FIG. 4. Rough colonies of Type IV *N. intracellularis* evenly sown on blood agar plate. $\times 11$.

FIG. 5. Minute discoid colonies of rough variant developed *in vitro* from a fresh Type II *N. intracellularis* strain. $\times 12$.

FIG. 6. Rough and smooth colonies of a fresh Type II *N. intracellularis* strain undergoing dissociation *in vitro*. $\times 12$.

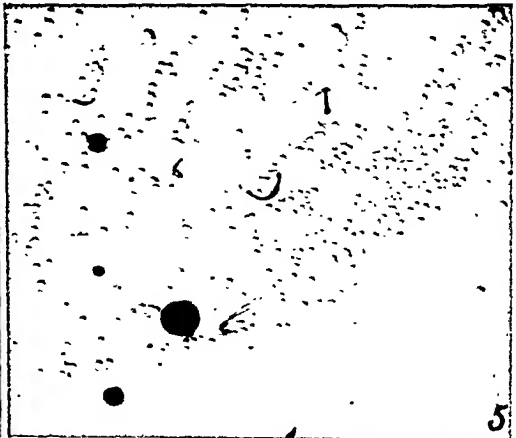
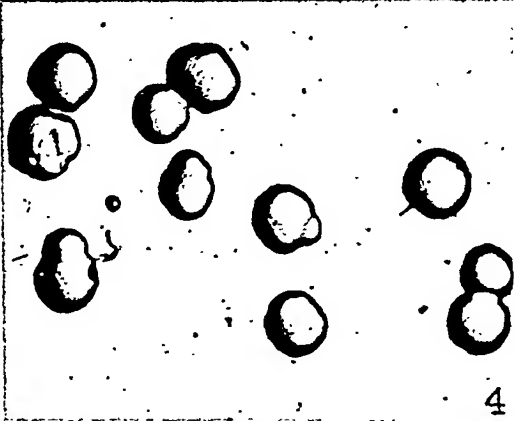
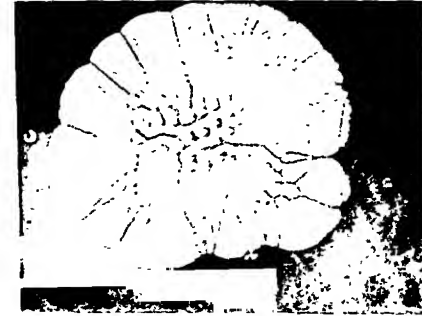
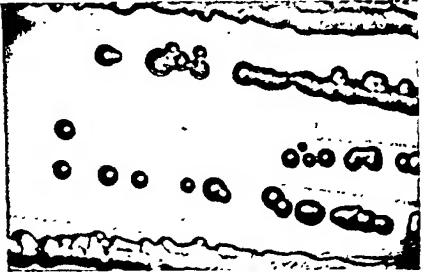
and determined directly on withdrawal from the water bath and without the overnight interval; and in the second, the results at this lower temperature are, as has been pointed out already, more delicate and free to a very large extent from the confusion caused by cross-agglutination. In this connection it may be said that the experience in this laboratory on applying the amended method of agglutination to strains of meningococci isolated from the cerebrospinal fluid has been at variance with that reported elsewhere. It is not unusual to find the statement made that the grouping of such meningococcus strains is by no means sharply defined. While this may be true with agglutination tests made at 56°C., all smooth cerebrospinal fluid strains tested at 37°C., 57 in number, have shown sharply defined grouping, if no distinction between Types I and III be made (see footnote 2).

It must be emphasized here that, although the agglutination method herein described is more satisfactory than that in which the test is run for 24 hours at 56°C., yet it is not put forward with the suggestion that it become the standard diagnostic method. The rapid method described by Nicolle, Debains and Jouan (8), by which agglutination can be read within 5 minutes, has all the advantages of rapidity and simplicity. The agglutination tests and agglutinating sera mentioned in this paper have appeared as corollaries during the development of a type-specific precipitating serum and are presented here as additional evidence of the differences between fresh and stock strains.

Further uses of the monovalent sera prepared with freshly isolated strains will be developed in subsequent papers which deal with the type-specific substances of the organisms and with the presence of meningococcus precipitinogens in the cerebrospinal fluid in cases of meningococcal meningitis.

SUMMARY

The production of monovalent sera for agglutinin or precipitin reactions with freshly isolated strains of meningococci is described. Agglutination reactions with such sera can be carried out more rapidly, at lower temperatures and in lower dilutions, than with the standard monovalent sera prepared from stock cultures, while the results so obtained are more satisfactory owing to the relative absence of cross-agglutination.



Photographed by Lou Schmidt

(Rake: Meningococcus infection. I)



STUDIES ON MENINGOCOCCUS INFECTION

II. MONOVALENT DIAGNOSTIC SERA PREPARED FROM "FRESH" AND "STOCK" STRAINS

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(Received for publication, November 10, 1932)

The preceding paper has dealt with certain morphological and biological differences demonstrable between fresh and stock strains of the meningococcus. These differences have further been found to be associated with differing antigenic qualities in the two strain varieties when they are used in the preparation or the testing of antimeningococcal sera and this paper deals with the preparation of sera from freshly isolated strains for use in agglutination and precipitation tests. The results of agglutination tests with such sera under different conditions are compared with those obtained by the use of standard monovalent sera prepared by immunization with stock strains of meningococci.

Material and Technique

The strains used included old stock strains, fresh spinal fluid and nasopharyngeal strains, and rough variants appearing *in vivo* or *in vitro*.¹ Standard monovalent diagnostic sera prepared with old stock strains have been procured through the courtesy of the New York State Department of Health, Albany. New sera have been prepared in this laboratory with freshly isolated strains (except for Type IV, of which no freshly isolated strain has been available), using young healthy rabbits.

Two methods of serum preparation have been used. In the first, rabbits are given four injections of gradually increasing numbers of living organisms on alternate days, the initial dose being from 1,000,000,000 to 2,000,000,000. Freshly

¹ The sources of these strains are given in Paper I of this series and will not be repeated here. It is desired, however, to express thanks again to all who have so kindly assisted in the difficult task of obtaining material for this work.

dextrose broth. Ayers and Rupp (2) later showed that the former group was further distinguished by the capacity of its members to hydrolyze sodium hippurate, whereas hemolytic streptococci from human sources did not split this substance.

Brown, Frost, and Shaw (3) concluded that no single test could be relied upon to differentiate the hemolytic streptococci commonly found in milk from the so called *Streptococcus epidemicus*. They were of the opinion, however, that probably the most constant and reliable of the tests tried by them were (a) the final hydrogen ion concentration attained in dextrose broth, and (b) the hydrolysis of sodium hippurate, although apparent exceptions were found to both. Most of the seven groups studied by them were differentiated by these two tests combined with fermentation reactions. They point out, however, that they encountered exceptional strains of bovine origin, characterized by a final pH of about 4.7 in dextrose broth and by little or no hydrolysis of sodium hippurate. These formed an interesting group intermediate between the other strains derived from cattle and those isolated from man. This group had not been described previously.

Streptococci of bovine origin culturally similar to this last group were found in considerable numbers by Minett and Stableforth (4), who concluded that these "streptococci were distinct from the usual udder streptococci and indistinguishable as a group by bacteriological and serological tests from *Streptococcus pyogenes* of direct human origin. The evidence therefore strongly suggests, though it cannot be held to prove, that in these cases the streptococci had been implanted in the udder by the milker." They also state that, although there was an absence of any definite disease among persons in contact with these infected cows, disease does not invariably occur among such persons even when the udder harbors streptococci probably pathogenic for man. These authors, furthermore, give a review of the literature indicating that similar strains were found by J. Smith (5), by Seelemann and Hadenfeldt (6), and by Diernhofer (7), all of whom considered them either closely related to *Streptococcus pyogenes* from man or indistinguishable from strains of human origin.

Edwards (8) described a group of strains from bovine and other animal sources which seemed to possess the same cultural characteristics as those studied by the above mentioned investigators. These strains did not hydrolyze sodium hippurate and were intermediate in their final pH range between the ordinary strains from bovine sources and those of human origin. Among the strains of animal origin studied by him, 94 per cent could be distinguished from those of human origin by the fact that they fermented sorbitol but not trehalose, while the strains from human sources did not ferment sorbitol but fermented trehalose. The remaining 6 per cent of strains derived from animals were, however, similar to the strains from human sources in their fermentation of these substances.

In addition to the streptococci classified by the above authors on the basis of biochemical and cultural reactions, R. C. Avery (9) showed that hemolytic streptococci derived from cheese and similar sources form still another group characterized by the capacity to reduce methylene blue and to grow in a medium containing concentrations of the dye which completely inhibit the growth of strains of human

temperature. For antigen a standard suspension has been adopted consisting of a suspension of meningococci of a sufficient density to give a reading of 4 cm. when tested with a Gates turbidometer and corresponding to about 2,000,000,000 organisms per cc. 0.3 cc. of this suspension is added to an equal amount of the serum or serum dilution to be investigated, the two fluids are mixed and the rack of tubes is placed in the appropriate water bath for the required length of time. When a temperature of 37°C. is used the tubes are removed after 2 hours and left in the ice box overnight, when a final reading is taken. The reading of the results must be largely a personal matter. In order to compensate for this, readings have been made by the same person throughout. A customary set of symbols has been used; namely, ++++ = complete agglutination; +++ = almost complete; ++ = definite; + = slight; and \pm = very slight. A control tube with saline and suspension in equal parts is always inserted to detect salt sensitivity. When agglutinations are done at 56°C. for 24 hours or longer, a second control tube of normal serum diluted 1/20 is added, but this has been found unnecessary at the lower temperature.

RESULTS

In Tables I and II are shown a comparison of both standard and "anti-S" (*i.e.* prepared with recently isolated strains) sera at both 37°C. and 56°C. when tested on three stock and three freshly isolated strains of Types I, II and III. Type IV will not be included in the following observation owing to the absence of any freshly isolated strains of this type.

Employing the anti-S sera at 37°C., it will be noticed that marked agglutination is obtained with homologous serum and that no cross-agglutination occurs.² Stock strains do not agglutinate as satisfactorily with these sera at the low temperature. It will be seen that while the type can be ascertained in all of the stock strains, the aggluti-

² The cross-agglutination between Types I and III must be regarded in another light. While it seems certain that in the past these two types, although antigenically similar, have been nevertheless distinct entities, the examples occurring at the moment show so much cross-agglutination that it is well nigh impossible to separate them into two types, and the term Group I-III has been adopted for all these strains. It has also been noticed both in this laboratory and elsewhere (2, 3) that the old stock strains of these two types, though antigenically distinct when first isolated, have now become so similar as to be almost indistinguishable (*vide* Strains 123 and 57 in Table I). Finally, it may be pointed out that the soluble specific substances from either of these types have proved to be indistinguishable by any of the methods adopted (4).

cocci. When, however, streptococci from a wider variety of animals were studied, the carbohydrate C, characteristic of human strains, was not found in strains from sources other than man, although it had been found in every strain of human origin previously examined, with a single exception reported by Hitchcock (12). This apparent absence of C from hemolytic streptococci isolated from lower animals led to investigations to determine whether similar substances might be found to differentiate other groups among these strains.

In order to avoid confusion, the existing information concerning the antigenic structure of hemolytic streptococci is summarized.

As far as chemical analysis of the immunologically differentiated substances is concerned, only strains of human origin have been studied previously. The members of this group contain (a) a type-specific protein "M," which differentiates types in agreement with the results obtained by agglutination and mouse protection tests (13 b, e); (b) a non-specific nucleoprotein, "P," which gives cross-precipitin and complement fixation reactions with similar fractions from related Gram-positive cocci, namely *Streptococcus viridans*, *Pneumococcus*, and *staphylococcus* (13 a); (c) another non-type-specific protein, "Y," about which little is known (13 c, e); (d) the non-type-specific protein, "Fraction D," of Heidelberger and Kendall (14) which they suggest may be the same as the Y fraction just mentioned; and (e) a non-type-specific carbohydrate, "C" (11, 12), which was formerly thought to characterize hemolytic streptococci and to be common to all strains.

From these cell constituents identified in hemolytic streptococci of human origin, only the carbohydrate C is of special significance in the present study because upon it this classification is based.

Methods

1. Immune Sera.—Rabbits were immunized with formalinized cultures as follows: The bacterial sediment from an 18 hour broth culture was suspended in one-twentieth volume of 0.85 per cent sodium chloride solution to which formalin was added in a final concentration of 0.2 per cent. After 48 hours in the ice box these bacterial suspensions were sterile. Immediately before use they were diluted with physiological salt solution to the original volume of the culture. Daily intravenous injections of 1 cc. were given for a week, followed by a week's rest. Two to four series of injections were made. Although good antisera were often obtained after two series, four or more courses were sometimes required. On the 5th day after the last injection test bleedings were made and the serum of animals showing a good titer was collected and stored in the ice box without a preservative.

All strains tried gave usable antisera by this method, although some were better antigens than others. After serial subcultures in 10 per cent type-specific immune

TABLE II

Strain	Standard monovalent serum																								Remarks	
	Type I								Type II								Type III									
	1/20	1/50	1/100	1/200	1/300	1/400	1/500	1/1,000	1/20	1/50	1/100	1/200	1/300	1/400	1/500	1/1,000	1/20	1/50	1/100	1/200	1/300	1/400	1/500	1/1,000		
Fresh Type I No. 17.....	±	±	±	±	±	±	0	0	±	±	±	±	±	±	±	0	±	±	±	±	±	±	±	±	0	Agglutination at 37°C. for 2 hrs. and ice box overnight
Fresh Type II No. 31.....	±	±	±	±	±	±	0	0	±	±	±	±	±	±	±	0	±	±	±	±	±	±	±	±	0	
Fresh Type III No. 7.....	±	0	0	0	0	0	0	0	±	±	±	±	±	±	±	0	±	±	±	±	±	±	±	±	0	
Stock Type I No. 123.....	1	2	1	1	1	1	1	1	±	±	±	±	±	±	±	0	1	1	2	1	±	±	±	±	±	0
Stock Type II No. 383.....	±	0	0	0	0	0	0	0	±	±	±	±	±	±	±	0	±	±	±	±	±	±	±	±	0	
Stock Type III No. 57.....	±	±	±	1	1	±	±	±	±	0	±	±	±	±	±	0	1	±	±	±	±	±	±	±	0	
Fresh Type I No. 17.....	1	1	2	1	1	1	1	±	0	1	1	0	0	0	0	0	1	1	2	1	1	±	±	±	0	Agglutination at 56°C. for 24 hrs.
Fresh Type II No. 31.....	1	1	±	±	0	0	0	0	1	1	±	±	±	±	±	0	±	±	±	±	±	±	±	±	0	
Fresh Type III No. 7.....	1	2	3	2	±	±	±	0	0	1	±	0	0	0	0	0	1	2	2	2	2	±	0	0	0	
Stock Type I No. 123.....	1	2	3	3	3	3	2	1	1	1	±	0	0	0	0	0	1	2	2	2	2	2	1	1	1	
Stock Type II No. 383.....	±	±	0	0	0	0	0	0	1	1	±	±	±	±	±	0	1	1	0	0	0	0	0	0	0	
Stock Type III No. 57.....	1	2	3	3	3	2	2	1	1	2	1	1	±	±	±	0	1	2	3	3	3	3	2	1	1	

4. *Final pH*.—The final pH attained in 1 per cent dextrose broth was determined colorimetrically essentially by the method of Avery and Cullen (1). Readings were made at the end of 4 days' incubation. Some of the first readings were made in duplicate with methyl red and brom-cresol green as indicators, but the later readings were all made with brom-cresol green since with this indicator the color changes were easier to distinguish.

5. *Hydrolysis of Sodium Hippurate*.—This test was made according to the method of Ayers and Rupp (2). The cultures were grown 4 days in infusion broth containing 1 per cent of sodium hippurate; then to 1 cc. amounts of the clear supernatant culture fluid were added 0.3, 0.4, and 0.5 cc. respectively of 12 per cent ferric chloride containing concentrated hydrochloric acid in the proportion of 2.5 cc. per liter. Uninoculated sodium hippurate broth incubated for the same period was used as a control, and sufficient ferric chloride was used in the test to insure complete clearing of the uninoculated control. Care was taken to shake the tubes thoroughly as soon as the ferric chloride was added. If the reaction was positive, a heavy precipitate of ferric benzoate was formed which was insoluble in the excess of ferric chloride. If the reaction was negative, the hippurate and protein precipitates formed at first were redissolved in the ferric chloride leaving a clear solution. Known positive and negative cultures were included as controls in every series tested.

6. *Reduction of Methylene Blue Milk*.—The method of R. C. Avery (9) was used for this test as follows: A 1 per cent solution of methylene blue was added to sterile milk to make a final concentration of 1:5000. 0.1 cc. of fresh 18 hour broth culture was inoculated into 5 cc. of methylene blue milk and into control tubes of milk without the dye. Readings were made after 24 hours' incubation and at intervals up to a week. The 24 hour readings are recorded in the tables, and the few changes occurring later are also noted.

7. *Growth on Blood Agar Containing Bile*.—Rabbit blood agar plates containing 10 or 40 per cent ox bile (10) were used to test the ability of these strains to grow in the presence of bile. Control plates of blood agar without the addition of bile were always inoculated at the same time. The 24 hour readings recorded in the tables were essentially the same as observations made after 3 or 4 days' incubation.

8. *Fermentation Reactions*.—1 per cent trehalose and sorbitol were added respectively to tubes of Hiss serum water with Andrade's indicator. Cultures were observed at intervals, and final readings recorded at the end of a week's incubation (8). Where neither substance was fermented and in case of doubtful growth, subcultures were made on blood agar plates to determine the presence of viable organisms. When tests with both substances were negative, a second set of tubes was inoculated.

9. *Lysis with Streptococcus Bacteriophage*.—These tests were performed by adding 1 cc. of fresh 18 hour blood broth culture (without disturbing the sedimented blood) to 5 cc. of plain broth and incubating for from 2 to 3 hours. To 1 cc. of such a culture 1 cc. of bacteriophage was added. This bacteriophage was derived

change with prolonged culture on artificial media. Strains tend to lose what narrow specificity they originally possess and to "broaden out" until they agglutinate markedly in heterologous as well as homologous sera. More important still is the fact that, as has been shown (5, 7), these stock strains have lost at least a large part of their specific substance on which antigenic specificity depends. The results of the work here reported strongly favor the view that freshly isolated strains give rise to sera which differ quantitatively, if not qualitatively, from those prepared with stock strains. It has not been possible for the writer to produce sera with stock strains which give results as satisfactory, as those obtained with the fresh strains, whether the sera be used for agglutination or precipitation tests. In fact, no precipitins for the specific fraction of the organisms have yet been obtained in sera prepared with stock strains.

It is generally agreed that the titre of agglutinins, which a serum should attain in order to be of value, must be high, 1/1,000 or more. With the standard sera generally used and the methods of agglutination usually adopted, it is not unusual for cross-agglutination to occur at a dilution of 1/100 or higher, and high titre sera are necessary in order to obviate any confusion in typing. As has been shown in the body of the present paper, however, such high titres are unnecessary when the sera are prepared with fresh strains and the reaction is carried out at 37°C. for a short period of time. Under these circumstances, a maximum titre of 1/100 will suffice and cross-agglutination is unusual even at the lowest dilution, 1/10. Since the amended method calls for the use of sera with only a low titre, it is possible to produce these in a comparatively short time and with few injections. This fact of itself is favorable to the production of narrowly specific sera, for rabbits injected repeatedly with a given strain yield a serum of steadily increasing non-specificity.

Using standard sera prepared with stock strains, it has been found necessary to perform the agglutinations at a temperature of 56°C., and the tubes have to be left at this temperature for 24 hours or longer before a conclusive result can be obtained. That the amended method calls for the use of a temperature of only 37°C. for only 2 hours, followed by the ice box overnight, is of double advantage. In the first place, the method is more rapid since agglutinations can usually be read

TABLE I

Specific Precipitin Reactions of Representative Strains from the Five Groups Differentiated by Serological Methods

Cultures					0.2 cc. antiserum prepared against strains of Groups				
Strain	Group	Source	Disease	Extract	A	B	C	D	E*
C 203	A	Man	Scarlet fever	cc.					
				0.4	+++	—	—	—	—
				0.1	++	—	—	—	—
				0.025	+	—	—	—	—
K 96	"	"	Pneumonia	0.4	++	—	—	—	—
				0.1	+±	—	—	—	—
				0.025	—	—	—	—	—
K 107	B	Cow	Mastitis	0.4	—	++	—	—	—
				0.1	—	+++	—	—	—
				0.025	—	++	—	—	—
K ₂ 126	"	"	None (certified milk)	0.4	—	++	—	—	—
				0.1	—	+++	—	—	—
				0.025	—	++	—	—	—
P 454	C	Guinea pig	Lymphadenitis	0.4	—	—	++	—	—
				0.1	—	—	+++	—	—
				0.025	—	—	++	—	—
K 150 A	"	Cow	Mastitis	0.4	—	—	+++	—	—
				0.1	—	—	++	—	—
				0.025	—	—	+	—	—
K 158 E	"	Rabbit	Pneumonia	0.4	—	—	+	—	—
				0.1	—	—	++	—	—
				0.025	—	—	++	—	—
K 155 A	"	Horse	Pleuropneumonia	0.4	—	—	+++	—	—
				0.1	—	—	++	—	—
				0.025	—	—	+	—	—
K 155 P	"	Swine	Abortion	0.4	—	—	+++	—	—
				0.1	—	—	+++	—	—
				0.025	—	—	++	—	—
K 155 N	"	Chicken	Slipped tendon	0.4	—	—	+++	—	—
				0.1	—	—	+++	—	—
				0.025	—	—	++	—	—

In the precipitin tests recorded in Tables I, II, and III, all volumes were made up to 0.6 cc. with saline. The tubes were incubated in a water bath at 37°C. for 2 hours and read after standing overnight in the ice box. Controls of serum with saline, and of extract with saline were all negative. The degrees of positive reaction are indicated by + to +++++; a negative reaction is indicated by —.

* The serum of one rabbit immunized with Strain K 129, Group E, gave slight cross-reactions with almost all extracts tested against it. Other rabbits of this series gave negative results as shown.

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TABLE II a
Serological Grouping and Summary of Biochemical and Cultural Characteristics of All Strains
Group A: Strains Chiefly of Human Origin

Origin of cultures			Serological reactions				Biochemical and cultural reactions							
Strain	Source	Disease	Precipitin tests				Final pH in dextrose broth	Hydrolysis of sodium hippurate	Reduction of methylene blue milk	Growth on bile agar		Fermentation		Lysis with streptococcus bacteriophage
			Antisera for Groups							10 per cent	40 per cent	Trehalose	Sorbitol	
			A	B	C	D								
1	N. Y. 5	Scarlet fever	+	-	-	-	5.0	-	-	++	+	+	-	-
2	C 203	"	+	-	-	-	5.0	-	-	-	+	+	-	-
3	O 89	"	+	-	-	-	4.8	-	-	++	+	+	-	-
4	K 151 D	"	+	-	-	-	5.1	-	*	-	+	+	-	-
5	K 151 E	"	+	-	-	-	5.1	-	-	-	+	+	-	-
6	K 96	Pneumonia	+	-	-	-	5.0	-	-	-	+	+	-	-
7	K 138	"	+	-	-	-	5.0	-	-	-	+	+	-	-
8	K 139	"	+	-	-	-	5.0	-	-	-	+	+	-	-
9	K 148	"	+	-	-	-	5.2	-	-	-	+	+	-	-
10	K 169	"	+	-	-	-	5.0	-	-	-	+	+	-	-
11	K 109	Infected gland	+	-	-	-	5.0	-	-	-	+	+	-	-
12	K 152	Lung infarct	+	-	-	-	5.0	-	*	-	+	+	-	-
13	K 197	Meningitis	+	-	-	-	5.0	-	-	-	+	+	-	-
14	M 34	Tonsillitis	+	-	-	-	5.2	-	-	-	+	+	-	-
15	K 210	Peritonsillar abscess	+	-	-	-	5.0	-	-	-	+	+	-	-
16	Boston	Septic sore throat	+	-	-	-	5.2	-	-	-	+	+	-	-
17	K 151 B	None†	+	-	-	-	5.0	-	-	-	+	+	-	-
18	K 151 C	" †	+	-	-	-	5.0	-	-	-	+	+	-	-
19	K 171 E	"	+	-	-	-	5.0	-	-	-	+	+	-	-
20	K 171 F	"	+	-	-	-	5.0	-	-	++	+	+	-	-
21	K 171 G	"	+	-	-	-	5.2	-	-	++	+	+	-	-
22	K 158 C	Spontaneous infection	+	-	-	-	5.2	-	-	++	+	+	-	-
23	V 10†	Mastitis (epidemic of septic sore throat)	+	-	-	-	5.1	-	-	++	+	+	-	-

A SEROLOGICAL DIFFERENTIATION OF HUMAN AND OTHER GROUPS OF HEMOLYTIC STREPTOCOCCI

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The differentiation of hemolytic streptococci from various animal sources has been a problem presenting many difficulties. Although numerous biochemical and cultural methods of differentiation have been advanced for the solution of this problem, it is apparent that a satisfactory serological test would be of considerable value. The object of the present paper is to describe a precipitin reaction which differentiates hemolytic streptococci into several groups. The proposed classification is based upon the study of 106 strains of *Streptococcus haemolyticus* isolated from a wide variety of sources: man, other animals, and dairy products. The results of this study are of interest not only from the theoretical viewpoint of establishing an orderly grouping of these microorganisms, but also from an epidemiological aspect in providing a means of identifying the probable origin of a given strain. The biochemical and cultural methods previously employed furnish presumptive evidence of the epidemiological importance and probable source of the strains in question. However, strains are occasionally encountered which are difficult or impossible to classify. Certain of the difficulties inherent in these methods appear to be largely overcome by the use of the precipitin test as a means of differentiation.

Particular attention has been paid by many investigators to the identification of hemolytic streptococci of human origin and to the separation of these strains from those ordinarily associated with bovine mastitis, or normally present in milk and dairy products. Avery and Cullen in 1919 (1) were able to differentiate clearly between one group of strains of bovine origin and those of human origin by determining the final hydrogen ion concentration of cultures grown in 1 per cent

classification based on the anti-C precipitin reaction. The characteristics of the groups defined are the following:—

Group A.—Extracts of the 23 strains placed in Group A gave positive precipitin reactions with antisera prepared against any member of the group, and gave no cross-reactions with antisera for any other group. The classification made on this basis was confirmed by additional biochemical and cultural tests. The strains of Group A, with two exceptions, were all derived from human sources.

One exceptional strain, V 10, came from the udder of a cow suffering from mastitis and was isolated during one of the Massachusetts epidemics of septic sore throat by Smith and Brown (17), and studied later by Avery and Cullen (1). Both groups of authors considered it a strain primarily of human origin and thought that the cow had probably become infected through a milker. The other strain in Group A, not of human origin, was isolated from a spontaneous lung infection in a rabbit. Whatever may be the true explanation of such exceptions, it is obvious that an overwhelming majority of strains in Group A are of human origin.

Turning now to groupings made on the basis of biochemical and cultural characteristics, quite good agreement with the classification by serological means is found.

When judged by the two tests principally recommended by Brown, Frost, and Shaw, namely final pH in dextrose broth and hydrolysis of sodium hippurate, it is seen that all the strains of Group A reacted with respect to these tests in the manner characteristic of strains of human origin. One strain gave a final pH of 4.8, while the rest ranged between pH 5.0 and 5.2, and none hydrolyzed sodium hippurate. None reduced methylene blue milk within 24 hours, although two strains gave growth and evidence of reduction after 3 days' incubation. These two exceptional results may have been dependent on the size of the inoculum, a factor stated (6) to be of considerable importance in the reaction. A few strains of this group grew on 10 per cent bile blood agar, and one even showed slight growth on 40 per cent bile blood agar. Minett and Stableforth (4) also found a few strains isolated from human sources with which growth was not inhibited on this medium.

The differential fermentation of trehalose and sorbitol was tested since it was advocated by Edwards (8) as a means of distinguishing between hemolytic streptococci of human derivation and those strains of animal origin which resemble human strains in not hydrolyzing sodium hippurate. All the members of Group A fermented trehalose and none fermented sorbitol, which is in agreement with Edwards' findings.

Of the 23 strains in Group A only six were found partially susceptible to the action of streptococcus bacteriophage and not very markedly so.

Group B.—The 21 members of Group B were classified together on the basis of their common precipitin reactions. The only cross-

and bovine origin. Seelemaun and Hadenfeldt (6) later called attention to the importance of the size of the inoculum in this test, as some strains ordinarily inhibited in this medium grow in it if a sufficiently large inoculum is used, and, as a result of growth, reduce the methylene blue.

Minett and Stableforth as well as Seelemaun and Hadenfeldt, using a test introduced by Belenky and Popowa (10), also showed that the hippurate-hydrolyzing bovine strains grow on blood agar containing 40 per cent bile, whereas human strains and the non-hippurate-hydrolyzing bovine strains only grow to a slight extent on 10 per cent, and rarely on 40 per cent bile blood agar.

Although these and other biochemical and cultural tests have been useful in the differentiation of hemolytic streptococci from various sources, a serological test would add considerable weight to conclusions regarding the origin of a particular strain. The agglutination reaction has heretofore proved unsatisfactory for this purpose on account of (a) the troublesome spontaneous agglutination so commonly encountered among streptococci, (b) the non-specific cross-agglutination, difficult of interpretation, and (c) the existence of so many different specific types as to make identification of strains impractical by type-specific agglutination. In addition to these difficulties, the agglutination reaction has been found inadequate for group classification in the present study because it does not disclose the specific groupings revealed by the results of the precipitin reaction. This lack of parallelism between the agglutination and precipitation tests may, in part at least, be attributable to the distribution within the cell of the various constituents. An insufficient concentration of the specific group antigen at the surface of the bacterial body might prevent the union of this antigen with its antibody with the result that group agglutination would not occur. When, however, the cell constituents are extracted and in solution, the reaction between the group-specific substance and its antibody is no longer masked.

These obstacles can be overcome to a large extent by employing the precipitin reaction. This test is apparently dependent on the presence in streptococci of substances characteristic of the large groups although not specific for the types within the groups. For some years it has been known that hemolytic streptococci of human origin contain a carbohydrate, the so called C substance, which is not type-specific (11); and this was previously thought to be identical in all hemolytic strepto-

reactions between members of this group and antisera for other groups were a few slight disc-like precipitates observed with extracts of two strains.

Strain M 216 gave a cross-precipitin test with Group C antiserum in addition to the much stronger major reaction with Group B antiserum. Strain K 158 A gave a cross-reaction with Group D antiserum, but here again the reaction with Group B antiserum was much more marked. In both instances all other characteristics were typical of Group B. The strains of this group were all similar in their biochemical and cultural characteristics. They fermented dextrose in broth to a final pH of 4.4 to 4.6; all hydrolyzed sodium hippurate; they did not reduce methylene blue milk; they grew actively on blood agar containing even 40 per cent bile; they were similar to strains of Group A in fermenting trehalose but not sorbitol; and they were not susceptible to streptococcus bacteriophage.

Group B, comprising 21 strains chiefly of bovine origin, corresponds to the group called *Streptococcus mastitidis* by many authors. In this series two strains isolated from man and one from a rabbit also fell into this group on the basis of the precipitin test, a finding confirmed in all three instances by the cultural and biochemical characteristics. One of these, Strain K 198, was obtained in almost pure culture from the throat of a child who had been exposed to scarlet fever. Neither at the time of taking the throat culture, nor subsequently, did she develop any symptoms of the disease, and it was ascertained that she had always drunk unpasteurized certified milk.

There is some uncertainty concerning the source of Strain O 90, another exceptional member of this group, in that it also has a history of human origin. Dr. E. W. Todd, who furnished the culture, states (18) that it is an encapsulated streptococcus coming originally from Dr. Wamoscher as one of Aronson's scarlatinal strains. Dr. Todd also sent us the strain "Aronson Schnitzer," with the note that it was entirely different from the other Aronson strain. "Aronson Schnitzer" (O 89 in our series) fell into Group A, while "Aronson Wamoscher" (O 90 in our series) had all the characteristics of Group B. Strain O 90 could, in fact, be identified as a member of this group both by its cultural and biochemical characteristics and by the specific precipitin reaction characteristic for this group.

No. K 158 A, the third strain in this group not isolated from cattle or milk, was obtained from a rabbit which had been used in the laboratory for testing vaccine virus.

Group C.—The members of Group C were derived from a variety of animal sources other than man. This group of 49 strains distinguished by the specific anti-C precipitin reaction, is largely composed of strains which have, by other methods, been difficult, or at times impossible, to differentiate from hemolytic streptococci of human origin. By

serum, the resulting culture was relatively devoid of type-specific substance and proved the best antigen for inducing the formation of anti-C precipitins specific for each group.

Antisera for the strains of human origin (Group A) were chiefly those which had been prepared previously by a method already described employing increasing doses of heat-killed organisms followed by living culture (13c) although the method noted above was satisfactory for these strains also. With many of the strains of the other groups it was impossible to use a scheme of immunization necessitating the injection of living culture, since too great a loss of animals resulted.

Antisera were tested with extracts of both homologous and heterologous strains of the same group in order to make sure of the presence of the group anti-C precipitin. The type-specific antibody for a subgroup, or type, was often present in addition to the group anti-C precipitin used in this classification, and was sometimes the only antibody present. Consequently if an extract of the homologous strain were the only one used in testing a serum, a type-specific reaction might be obtained which would mask the group, or anti-C, precipitin reaction, and the presence or absence of this anti-C precipitin might not be discovered. Since this classification is based on the anti-C precipitin reaction, it was essential in testing antisera to employ an extract of a strain of heterologous type but homologous group, as measured by the anti-C reaction.

2. *Extracts*.—Extracts were made by a method previously employed in preparing the type-specific substance, M, of strains of human origin (13b). The bacterial sediment from 250 cc. of an 18 hour broth culture was suspended in 5 cc. of physiological salt solution containing sufficient normal hydrochloric acid to make a final concentration of $N/20$ HCl. The reaction of the suspension was tested with Congo red paper and, if necessary, enough hydrochloric acid was added to turn the paper blue. The tube was then immersed in boiling water for 10 minutes, cooled under running water, and centrifuged. The supernatant fluid was neutralized, the resulting precipitate discarded, and the water-clear supernatant fluid was used in the precipitin test. Obviously, such a crude extract contained a mixture of substances, but these did not interfere with the reaction under consideration.

3. *Precipitin Test*.—In performing this test increasing amounts of extract were placed in a series of tubes; usually 0.4, 0.1, and 0.025 cc. constituted the series. The volumes were made up to 0.4 cc. with normal salt solution, and a control tube with 0.4 cc. of the same diluent was included. A constant volume of 0.2 cc. of undiluted antiserum was layered in each tube and allowed to stand for 10 to 30 minutes either at room temperature or in the water bath at 37°C. in order to observe ring formation. The tubes were then shaken and incubated for 2 hours at 37°C. in the water bath. Final readings were made after the tubes had stood overnight in the ice box. Sometimes a larger series of dilutions was employed, but no difference in result was obtained. Extracts from all strains were tested in the same way as those detailed in Table I, but the results of the precipitin test are condensed in Table II and the titration is merely recorded as positive or negative.

TABLE II C
Serological Grouping and Summary of Biochemical and Cultural Characteristics of All Strains
Group C: Strains Isolated from a Variety of Lower Animals

Origin of cultures			Serological reactions				Biochemical and cultural reactions							
Strain	Source	Disease	Precipitin tests				Final pH in dextrose broth	Hydrolysis of sodium hippurate	Reduction of methylene blue milk	Growth on bile agar		Fermentation		Lysis with streptococcus bacteriophage
			A	B	C	D				10 per cent	40 per cent	Trehalose	Sorbitol	
1	K 56	Lymphadenitis	-	-	+	-	4.7	-	-	++	-	-	+	+
2	K 57	"	-	-	+	-	4.8	-	-	-	-	-	+	+
3	K 58	"	-	-	+	-	4.8	-	-	+	-	-	+	+
4	K 59	"	-	-	+	-	4.9	-	-	+	-	-	+	+
5	K 60	"	-	-	+	-	4.8	-	-	+	-	-	+	+
6	K 61	"	-	-	+	-	4.8	-	-	++	-	-	+	+
7	K 62	"	-	-	+	-	4.7	-	-	+	-	-	+	+
8	K 64	"	-	-	+	-	4.8	-	-	+	-	-	+	+
9	P 230	"	-	-	+	-	4.7	-	-	±	-	-	-	+
10	K 104	"	-	-	+	-	4.8	-	-	+	-	-	-	+
11	K 159	"	-	-	+	-	4.8	-	-	++	-	-	+	+
12	K 132	"	-	-	+	-	4.7	-	-	++	-	-	+	+
13	P 451	"	-	-	+	-	4.8	-	-	++	-	-	+	+
14	P 546	"	-	-	+	-	4.8	-	-	++	-	-	+	+
15	K 171 A	"	-	-	+	-	4.8	-	-	++	-	-	+	+
16	K 171 B	"	-	-	+	-	4.8	-	-	++	-	-	+	+
17	K 171 C	"	-	-	+	-	4.7	-	-	+	-	-	+	+
18	K 171 D	"	-	-	+	-	4.8	-	-	+	-	-	+	+
19	K 106	Mastitis	-	-	±	-	4.6	-	-	-	-	-	+	+
20	K 150 A	"	-	-	+	-	4.7	-	-	++	+	-	+	+
21	K 150 B	"	-	-	+	-	4.9	-	-	++	+	-	+	+
22	K 150 C	"	-	-	+	-	4.8	-	-	-	-	-	+	+
23	K 150 D	"	-	-	+	-	4.8	-	-	++	-	-	+	+
24	K 150 E	"	-	-	+	-	4.8	-	-	++	-	-	+	+

from that isolated by Clark and Clark from sewage (15). Either the Berkefeld filtrate of a lysed culture, or the unfiltered culture, crystal-clear after lysis by the phage, was used in these experiments. The only difference observed in filtered or unfiltered reagent was that the filtered phage was somewhat less active. Controls of culture plus broth were also included. The tubes were incubated at 37°C. for from 1 to 18 hours; but lysis was usually complete in 1 hour if it occurred at all. Overgrowth of resistant forms was frequently observed if the tests were incubated overnight.

RESULTS

The specific groupings presented in the tables were made entirely on a serological basis according to the results of the anti-C precipitin test. Table I shows the details of a representative series of precipitin reactions typical of the results obtained with all the strains and recorded in Table II. A control series of precipitin tests with extracts prepared from cultures of *Streptococcus viridans* is given in Table III. The striking group specificity is obvious from Table I. Thus, precipitin tests with extracts of the two representative strains from Group A showed very similar titers with antiserum prepared against another strain of Group A, and did not give any cross-reactions with antisera prepared against hemolytic streptococci of the other four groups represented. The same relationship exists within the other groups: the members of each group react only with serum specific for that group. The correlation between the sources of the cultures and the specific groups into which the strains are differentiated serologically is also made evident in Table I. Group A is composed largely of strains of human origin; Group B is made up of strains derived from mastitis in cows and from normal milk; Group C contains strains from various lower animals; Group D comprises only strains obtained from cheese; and the few strains in Group E were all isolated from certified milk.

Of the 106 strains studied in this series, 104 were classified into five groups, as shown in Tables I and II; two strains in the series remained unclassified. Ten strains of *Streptococcus viridans* included as controls failed to react in any of the group-specific sera. It is reasonable to suppose that with a more extensive survey additional groups may be found, but the number and varied source of the strains studied are sufficient to establish the principle that broad distinct groupings of hemolytic streptococci can be made serologically. No significance can

The three remaining cultures highly susceptible to bacteriophage formed Group E; but a few considerably less susceptible strains were included in Group A, so that this test was not specific for any serological group or groups, although its close association with Groups C and E was noteworthy.

One series of cultures in this group, supplied by Dr. F. H. Fraser of the University of Toronto, is of particular interest and illustrates how useful this test may be. Strains K 171, A, B, C, and D, isolated from guinea pigs in a spontaneous epidemic caused by hemolytic streptococci, all proved to be members of Group C; while Strains K 171 E, F, and G, isolated at that time during routine examinations of the throats of healthy attendants, all fell into Group A, the group containing the larger majority of strains from human sources.

Group D.—Group D, a uniform group of eight strains all derived from cheese, were among those used by Avery and Cullen (1) in their study of the final hydrogen ion concentration attained by hemolytic streptococci.

These strains attained a final pH in dextrose broth of 4.2 to 4.3; they hydrolyzed sodium hippurate, but only to a slight extent; they grew readily in methylene blue milk and reduced it overnight; they grew luxuriantly on blood agar containing as much as 40 per cent bile; they were variable in their fermentation of trehalose and sorbitol, although six of the eight strains examined fermented both; and they were not susceptible to lysis by streptococcus bacteriophage.

Group E.—Group E comprised three strains isolated by Dr. J. H. Brown from certified milk. They were members² of Groups 3 and 6 described by Brown, Frost, and Shaw (3). On preliminary examination they were thought to be members of Group C of this series because one of them, K 131, showed some cross-reaction with Group C antisera and because all three were very hemolytic on blood agar plates and also markedly susceptible to streptococcus bacteriophage. Further work showed that the precipitin reaction of Strain K 131 with Group C antisera was a minor one, not exhibited by the other two strains, and that antisera prepared against these three strains showed strong precipitin reactions with extracts of all three.

The antiserum from one rabbit immunized with Strain K 129 showed traces of cross-reactions with almost all extracts tested. This was not evident with the antiserum from another rabbit; hence it was felt that this difference had to do with an individual rabbit variation rather than with antigens contained in this strain.

² See footnote (*) to Table II *d*.

TABLE I—*Concluded*

Cultures					0.2 cc. antiserum prepared against strains of Groups				
Strain	Group	Source	Disease	Extract	A	B	C	D	E*
K 155 G	C	Fox	Pneumonia	cc.					
				0.4	—	—	+++	—	—
				0.1	—	—	+++	—	—
				0.025	—	—	++	—	—
C 6	D	Cheese	None	0.4	—	—	—	++	—
				0.1	—	—	—	++	—
				0.025	—	—	—	±	—
C 7	"	"	"	0.4	—	—	—	++	—
				0.1	—	—	—	++	—
				0.25	—	—	—	++	—
K 128	E	Cow	None (certified milk)	0.4	—	—	—	—	+++
				0.1	—	—	—	—	++
				0.025	—	—	—	—	±
K 129	"	"	"	0.4	—	—	—	—	+++
				0.1	—	—	—	—	++
				0.025	—	—	—	—	+

be attached to the relative proportions of the several groups since the strains other than those of human origin were chiefly obtained without reference to the frequency of their occurrence.¹

Because the so called anti-C reaction previously studied in members of Group A was found to be due to a carbohydrate, it seems probable that comparable substances of this nature are responsible for the specific reactions in the other groups. The marked group specificity of these substances is apparently an important factor in the notable lack of cross-reactions among groups. Another factor is the nature

¹ I am greatly indebted to Dr. O. T. Avery and Dr. E. G. Stillman, of The Rockefeller Institute Hospital; to Dr. Theobald Smith, Dr. F. S. Jones, and Dr. J. B. Nelson, of The Rockefeller Institute at Princeton, N. J.; to Dr. J. H. Brown, of the Johns Hopkins University; to Dr. W. N. Platridge, of Storrs Agricultural Experiment Station, Storrs, Connecticut; to Dr. P. R. Edwards, of the University of Kentucky; to Dr. E. M. Butt, of the University of Southern California; to Dr. F. H. Fraser, of the University of Toronto; to Dr. F. L. Mickle, of the Connecticut State Department of Health, Hartford, Connecticut; and to Dr. E. W. Todd, of the Belmont Laboratories, Sutton, Surrey, England; all of whom have kindly sent me cultures.

The cultural characteristics of this group were the following: the final pH attained in dextrose broth was 4.6 to 4.8; sodium hippurate was not hydrolyzed; methylene blue milk was not reduced; growth was not obtained on bile blood agar even when the concentration of bile was only 10 per cent; both trehalose and sorbitol were fermented; and the streptococcus bacteriophage caused rapid lysis of all three strains. It is not known how extensively this group is distributed, nor whether it is composed entirely of strains derived from milk, although the three classified here were isolated from certified milk.

Unclassified Strains.—Two strains were not classifiable in any of the above groups.

One of these, Strain K 130, from certified milk, was very similar culturally to the strains isolated from cheese. It was not as high an acid producer as the latter, reaching a final pH of only 4.6 in dextrose broth; in all other cultural and biochemical characteristics it was similar to members of Group D. Serologically, however, it was quite distinct. An extract prepared from a culture of Strain K 130 did not precipitate Group D antisera; and antisera prepared against Strain K 130 reacted with it alone out of the 106 strains in this series. A strong cross-reaction was observed between extracts of this strain and two antisera against strains of Group A, although other antisera prepared against the same strains at the same time showed no trace of cross-reaction. The homologous K 130 antiserum gave, however, the strongest reaction of all.

The other unclassified strain, K 208, of human origin, showed no hemolysis on the surface of blood agar plates until after 2 or 3 days' incubation. Deep colonies, however, showed definite zones of hemolysis after 18 hours' incubation, leaving no room for doubt that it was a beta hemolytic (21) type of streptococcus. This strain was isolated from a tooth abscess, and culturally was similar to the strains of Group A, although serologically it was different from all the strains for which antisera were available. No antiserum for this strain was prepared.

Streptococcus viridans.—Table III shows the results of the precipitin reactions of ten strains of *Streptococcus viridans* which were used as controls.

One of these, Strain K 157, was isolated from a guinea pig infection by Dr. Theobald Smith (20) and designated "Strain C" in his series, and the rest were from patients in hospitals. Strain A 84 showed a slight cross-reaction with a Group B antiserum, and Strains 38 D and K 157 showed a trace with a Group D serum, but all others tested were negative.

Other Cultural Characteristics.—In addition to the characteristics of the hemolytic streptococci listed in Table II certain others were observed.

of the crude extracts employed which, although composed of a mixture of active substances, nevertheless contained little or none of the protein fractions, such as P, because, by the method of extraction, most of the acid-precipitable and heat-coagulable proteins are removed from solution. In addition, the antisera were prepared in a manner calculated to prevent the formation of much protein antibody. Consequently, even with such a complex antigenic mosaic, only a minimal number of cross-reactions was observed. These occurred as disc precipitates and were usually so slight as almost to escape detection. The major reaction was generally strong enough to make the proper classification evident even without the confirmatory evidence of the biochemical reactions. Further work is necessary before the nature of this disc-like cross-reaction can be ascertained. The few exceptions to the group specificity commonly observed are indicated in Table II and are considered in detail in the discussions of the groups in which they occur.

In addition to a summary of the precipitin reactions, all of which were performed in the same manner as the tests recorded in Table I, Table II contains results showing the correlation between the serological reactions and certain of the cultural and biochemical reactions found of value by other investigators in differentiating hemolytic streptococci. The marked susceptibility to lysis by bacteriophage, exhibited by members of the two groups, C and E, and previously reported (16), is also recorded in Table II. The results of all these tests were used as confirmatory evidence of the validity of the present

Footnotes to Table II a

In Table II, + indicates a positive reaction, and - indicates a negative reaction. In the precipitin tests and the test for the hydrolysis of sodium hippurate, this is a condensed form and represents a titration using several dilutions. The degree of growth on bile agar is represented by + to + + + +. The details of the performance of all tests are given under Methods.

* Growth and reduction after 3 days' incubation.

† Isolated from healthy individuals during an epidemic of scarlet fever and septic sore throat in which K 151 D and K 151 E were obtained. All four are of the same serological type as Strain C 203 (22).

‡ Obtained from Dr. O. T. Avery. Considered to be a human strain by Avery and Cullen (1) as well as by Smith and Brown (17) who isolated it during an epidemic of septic sore throat.

mucoïd colonies. This was especially true of Strain P 230 of this series ("Strain A" of Dr. Theobald Smith (20) who isolated it from lymphadenitis in a guinea pig). This strain was highly virulent for rabbits, and passage through animals of this species was far more effective in restoring the mucoïd colony form and the accompanying high degree of virulence than passage through other animals. Blood agar cultures of these strains changed markedly in appearance after 2 or 3 days either at room temperature or in the incubator. The areas of hemolysis, which at first surrounded the individual colonies, soon merged until the whole sector on which the culture was plated became hemolyzed. At the same time the colonies became transparent and seemed to undergo lysis to a certain extent, although they did not entirely disappear. It was suggested in a previous paper (16) that this might be due to the presence of a bacteriophage associated with these cultures, especially since the majority of these strains were markedly susceptible to the action of a bacteriophage isolated from sewage (15).

The general question of virulence among the strains of the groups described was not systematically investigated, although incidental observations were made. The strains of Group A, largely of human origin, were usually of low virulence for laboratory animals, although in most instances this could be enhanced for a given species by repeated passage. Some of the strains of Group B were highly virulent for rabbits and mice, for example Strains O 90 and K 158 A; and most of those of Group C were virulent for these animals and for guinea pigs as well. It was, indeed, usually difficult to immunize rabbits with living cultures of members of this last group. No information in regard to virulence is available concerning members of the other groups.

DISCUSSION

The data presented in this paper show that hemolytic streptococci can be differentiated serologically by means of the precipitin reaction into distinct and sharply defined groups which are not disclosed by the agglutination reaction. The test is relatively simple and gives results which are strikingly uniform and consistent. The reagents essential in carrying out the test are heat and hydrochloric acid extracts of the microorganisms and the sera of rabbits immunized with formalinized cultures.

One hundred and four of the 106 strains of hemolytic streptococci in this study were classified into five groups, while each of the two remaining strains was different from all others in the series. Ten strains

TABLE II b
Serological Grouping and Summary of Biochemical and Cultural Characteristics of All Strains
Group B: Strains Chiefly of Bovine Origin

Group B; Strains Grouped by Bovine Origin

Origin of cultures			Serological reactions				Biochemical and cultural reactions							
Strain	Source	Disease	Precipitin tests				Final pH in dextrose broth	Hydrolysis of sodium hippurate	Reduction of methylene blue milk	Growth on bile agar		Fermentation		Lysis with streptococcus bacteriophage
			A	B	C	D				10 per cent	40 per cent	Trehalose	Sorbitol	
1 V 8	Bovine		—	+	—	—	4.4	+	—	+	+	+	+	—
2 V 9	"		—	+	—	—	4.4	+	—	+	+	+	+	—
3 C 69	"	Mastitis	—	+	—	—	4.5	+	—	+	+	+	+	—
4 K 107	"	"	—	+	—	—	4.4	+	—	+	+	+	+	—
5 K 151 A	"	Suspected mastitis	—	+	—	—	4.4	+	—	+	+	+	+	—
6 B 63	"	Acute	—	+	—	—	4.5	+	—	+	+	+	+	—
7 B 92	"	Suspected "	—	+	—	—	4.4	+	—	+	+	+	+	—
8 B 112	"	Mastitis	—	+	—	—	4.4	+	—	+	+	+	+	—
9 B 115	"	Chronic mastitis	—	+	—	—	4.6	+	—	+	+	+	+	—
10 B 116	"	Mastitis	—	+	—	—	4.4	+	—	+	+	+	+	—
11 B 120	"	"	—	+	—	—	4.5	+	—	+	+	+	+	—
12 B 125	"	"	—	+	—	—	4.5	+	—	+	+	+	+	—
13 B 126	"	"	—	+	—	—	4.4	+	—	+	+	+	+	—
14 B 132	"	"	—	+	—	—	4.6	+	—	+	+	+	+	—
15 B 135	"	Chronic mastitis	—	+	—	—	4.5	+	—	+	+	+	+	—
16 M 216	"	"	—	+	±	—	4.5	+	—	+	+	+	+	—
17 K 126	"	None	—	+	—	—	4.6	+	—	+	+	+	+	—
18 K 127	"	"	—	+	—	—	4.5	+	—	+	+	+	+	—
19 K 198*	Human	"	—	+	—	—	4.4	+	—	+	+	+	+	—
20 O 90†	"	Scarlet fever?	—	+	—	—	4.4	+	—	+	+	+	+	—
21 K 153 A	Rabbit	Vaccine virus test animal	—	+	—	±	4.4	+	—	+	+	+	+	—

* The child from whom this culture was obtained had always drunk unpasteurized certified milk.

† Obtained as Aronson scarlet fever strain but different from another strain (O 89) labelled "Aronson" which was furnished from another laboratory.

the occurrence in all the groups of chemically related but serologically specific substances. The group-specific substance found in strains of human origin has been identified as carbohydrate in nature. At present, however, no information is available concerning the chemical composition of the determinative substances which differentiate the other groups.³ Although the carbohydrate C, identified in members of Group A, was formerly thought to occur in all hemolytic streptococci, study of strains from different animal sources now indicates that other groups of hemolytic streptococci elaborate similar substances distinct from the C fraction of Group A and characteristic for each specific group. This is the basis of the serological test used in the present classification.

SUMMARY

1. All except two of 106 strains of hemolytic streptococci isolated from man, other animals, milk, and cheese have been classified into five groups, which bear a definite relationship to the sources of the cultures. These broad groups may be subdivided into specific types by methods discussed elsewhere. The specific group classification is made possible by employing two special reagents: (*a*) extracts prepared by treatment of the bacteria with hot hydrochloric acid, and (*b*) serum of animals immunized with formalinized cultures. This differentiation is not detected by the agglutination reaction. The grouping agrees with that described by other investigators on the basis of cultural and biochemical characteristics.

2. The group-specific substance present in strains of Group A has been identified chemically as carbohydrate in nature. The chemical composition of the specific substances upon which the specificity of the other groups depends has not been determined. It seems not unlikely, however, that all of them may belong in the general class of carbohydrates, each being chemically distinct and serologically specific in the individual groups.

³ At the time that proof was read (Feb. 25, 1933), additional data were available which show that the substance which determines the specificity of Group B is also carbohydrate in nature.

means of the precipitin reaction, however, the specific differentiation of this group is unequivocal. The single cross-reaction observed was a slight disc-like precipitate formed when an extract of Strain K 106 was tested with certain antisera for Group B, but not with all antisera.

All of the 18 strains isolated from guinea pigs belonged in this group together with most of those from rabbits, the majority of the strains of bovine origin which did not hydrolyze sodium hippurate, and all strains originating from diseased horses, foxes, swine, and chickens. The strains from the last four sources, as well as a number from cattle, were kindly supplied by Dr. P. R. Edwards. Streptococci of bovine origin which have been described as having cultural characteristics very similar to those of the strains in this group have caused several English and German investigators (4) to express doubt concerning the possibility of distinguishing them from strains of known human origin, although Dr. Edwards was able to differentiate 94 per cent of those he examined by means of the fermentation of two substances. By comparing the cultural and biochemical characteristics of this group with those of Group A it is easy to understand the possible difficulty in deciding definitely on the basis of these tests that strains of Group C are not of human origin. The members of this group had a final pH range in dextrose broth of 4.6 to 4.9, which was intermediate between the ranges of Groups A and B, and indeed, overlapped to some extent the pH ranges of these two groups. The strains of Group C were also similar to those of Group A in the following respects: they did not hydrolyze sodium hippurate nor did they usually reduce methylene blue in milk, although a few exceptions were observed after 48 hours' incubation; and only one strain grew on 40 per cent bile blood agar, but most of them grew more or less abundantly on blood agar containing 10 per cent bile. Forty-five of the 49 strains in this group fermented sorbitol and not trehalose, a characteristic distinguishing them from streptococci of human origin. Four strains did not ferment either of these substances. Two of these, K 155 B and K 196, were *Streptococcus equi* according to Holman's classification (19); and Edwards found that among the streptococci examined by him only *Streptococcus equi* failed to ferment either sorbitol or trehalose. In the present series, two strains isolated from guinea pigs (Strains P 230 and K 104) also left these substances intact, but differed from *Streptococcus equi* in fermenting lactose.

As reported in a previous paper (16), the majority of hemolytic streptococci susceptible to the bacteriophage originally isolated by Clark and Clark (15) were found in this group; and only two strains included in the group were not subject to lysis in these experiments. Of these, K 196 was tested on one occasion only, but K 155 L was tested repeatedly under the most favorable conditions. With this culture it is possible that resistant forms had entirely overgrown the non-resistant. This was suggested partly by the fact that growth of the culture on blood agar plates always occurred in the form of fine, dry colonies without mucoid characteristics. This growth was similar to that of a resistant subculture of the original susceptible strain (R 226).

[illegible]

* Reduced in 18 hours.

† These two strains are *Streptococcus equi*.

inspection when animals were sacrificed) next showed that whenever a marked increase in erosion took place, an increased gastric retention of test material was involved. Possible changes in gastric acidity were therefore more or less obscured by this complication, but data thus secured served very well for a study of the development of gastric retention and pylorospasm.

Methods

Inasmuch as the method chiefly used was primarily devised to obtain data concerning gastric erosion, it was not as simple as one which might have served to yield suitable data regarding gastric retention. After a few preliminary tests, a definite number of weighed pieces of metal were generally given to the rats three times daily—morning, afternoon and evening. Thus, from 50 to 200 pieces of metal of a single type would usually be given to each animal during a 3 day period. Then some other type of test metal would be given during the next 3 days, and so on. No type of test material was again given to a rat until all of the type previously given was recovered or otherwise accounted for.

The following first eight types of test material (steel, iron and aluminum) were used in the main study: (1) 1/16 inch (about 1.6 mm.) stainless steel balls, such as are used in ball bearings (Fig. 1). 200 of these, weighing about 3.2 gm., were generally given during 3 day periods. Before being used, the new steel balls were slightly eroded with HCl to break the smooth surfaces. (2) 1/16 inch ordinary or hardware grade steel balls. The weight of these and the amount given was about the same as in the case of stainless steel balls. This and other types of reused test material that tended to rust were always cleaned with HCl and dried with alcohol and ether before being weighed and given again to the rats. (3) Pieces of No. 15 (Brown and Sharpe gauge) soft iron wire, 2 to 3 mm. long, cut sheer with square-cutting pliers. 100 pieces, weighing about 3 gm., were usually given during 3 day periods. Before this type of material was given to rats, it had been given to rabbits in an attempt to produce gastrointestinal lesions by mechanical irritation (5). The material was consequently somewhat eroded and erosion of this type of material mainly caused pitting at the ends of the pieces. (4) Pieces of No. 15 soft iron wire about twice as long as the preceding, but with the ends rounded in a lathe. 50 pieces, weighing about 3 gm., were generally used in 3 day tests. (5) Pieces of No. 18 soft iron wire, cut sheer after the wire was first cleaned with emery cloth. 100 pieces, weighing about 2.4 gm., were given during 3 day periods. This was the only type of test material that was given only in the form of new material each time—other types were used repeatedly after recovery and cleaning, unless eroded too much. (6) Pieces of No. 18 aluminum wire about 4 mm. long. 100 pieces, weighing about 1.2 gm., were used in 3 day tests. All aluminum material was simply cut sheer with square-cutting pliers and was used after the smooth surface was broken by erosion with HCl *in vitro*. (7) Pieces of No. 16 aluminum

*Serological Grouping and Summary of Biochemical and Cultural Characteristics of All Strains
Group D, Group E, and Unclassified Strains*

REBECCA C. LANCEFIELD

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Origin of culture			Serological reactions					Biochemical and cultural reactions						
Strain	Source	Disease	Precipitin tests					Final pH in dextrose broth	Hydrolysis of sodium hippurate	Reduction of methylene blue milk	Growth on bile agar	Fermentation		Lysis with streptococcus bacteriophage
			Antisera for Groups									Trehalose	Sorbitol	
			A	B	C	D	E							

Group D: strains isolated from cheese

1	C 1	Cheese	—	—	—	—	—	—	—	+	+	+	—
2	C 2	"	—	—	—	—	—	4.2	±	+	+	+	—
3	C 3	"	—	—	—	—	—	4.2	±	+	+	+	—
4	C 4	"	—	—	—	—	—	4.3	±	+	+	+	—
5	C 5	"	—	—	—	—	—	4.3	±	+	+	+	—
6	C 6	"	—	—	—	—	—	4.3	±	+	+	+	—
7	C 7	"	—	—	—	—	—	4.2	±	+	+	+	—
8	C 8	"	—	—	—	—	—	4.2	±	+	+	+	—

Group E: three strains isolated from certified milk

1	K 128*	Bovine	—	—	—	—	—	—	—	—	—	—	+
2	K 129	"	—	—	—	—	—	4.8	—	—	—	—	+
3	K 131	"	—	—	—	—	—	4.6	—	—	—	—	+

Unclassified strains: one of bovine origin, one of human

1	K 130	Bovine	—	±?	—	—	—	4.6	+	+	+	+	—
2	K 208	Human	—	—	—	—	—	4.8	—	—	—	—	—

* K 128 of this series is "Certified Milk 21," Group 6, of Brown's series (3); K 129, "Certified Milk 10," Group 3, of Brown's series (3); K 131, "Certified Milk C 3056," Group 3, of Brown's series (3).

In addition to these strains, Dr. Brown kindly sent me "Certified Milk 4," Group 2, and a strain representing his Group 7, "Certified Milk 22-76" (Strains K 126 and K 127 respectively of this series), both of which fell into Group B serologically and culturally. Dr. Brown's strain, "Certified Milk 19," Group 5, is in this series an unclassified strain, K 130.

rats used in these tests developed gastric retention. This happened whether the diets were high in fat or high in carbohydrate (Text-figs. 1, 2 and 3), although diets high in carbohydrate were somewhat more effective here. Some of the variations in the results of protein restriction were evidently due to differences in the previous diets of the rats. Thus, Rat 916 (Text-fig. 1), which had previously been kept on an adequate stock diet, developed gastric retention only after a prolonged period of protein restriction, while Rat 866 (Text-fig. 2), which had been kept during the previous 3 months on diets low in fat and including large additions of kaolin and sand, developed gastric retention immediately after it was placed on a diet of white bread only; and this retention persisted when the diet was changed to one high in fat but still low in protein. In all (4) of the 7 rats in which we tried to produce gastric retention a second time with diets low in protein, after clearing up the first retention by protein realimentation, this proved to be possible.

In most cases, the gastric retention of metal did not develop until after the animals lost more or less weight and this might suggest that the retention was a consequence of a general weakening of the rats as a result of undernutrition. However, tests in which Rat 912 (Text-fig. 3) and another rat (Rat 911) were given only about 2 gm. daily of a diet adequate in its protein content (21 per cent) showed that gastric retention was not a consequence of simple undernutrition. This is in agreement with the finding of Menville, Ané and Blackberg (7) that the gastrointestinal rate in rats is accelerated by such semistarvation. It is also consistent with our observation that prepyloric lesions were rarely occasioned by starvation, although they frequently developed with diets low in protein (8). On the other hand, the onset or prolongation of gastric retention, when protein realimentation was attempted in the rats, suggests that severe undernutrition or prolonged protein restriction increased the irritability of the pyloric region, as was previously noted in man (9). Thus, Rat 909 (Text-fig. 2), which did not develop gastric retention during 42 days of protein restriction (2.5 per cent protein and 54 per cent fat), developed transient retention immediately after the diet was changed to one containing 63 per cent protein and 22 per cent fat. Likewise, Rat 912 (Text-fig. 3) showed transient retention with diets high in protein after periods of protein

The degree of hemolysis was fairly characteristic for each group. Thus, the members of Group B were the least hemolytic (about +), those of Group A considerably more hemolytic (about ++), those of Group D still more hemolytic (about +++ ±), and strains included in Groups C and E were the most markedly hemolytic (about ++++).

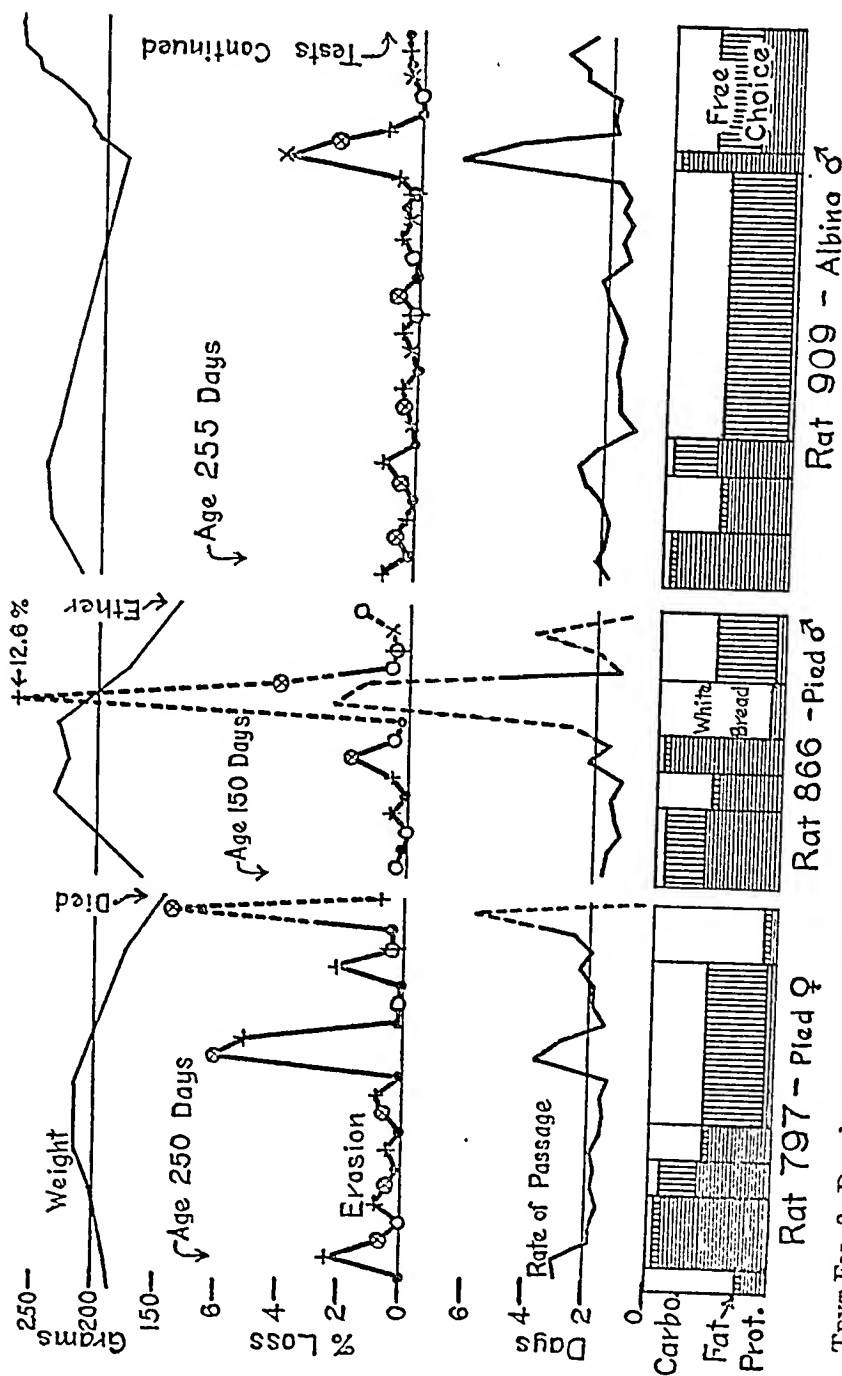
TABLE III
Precipitin Tests with Control Group of Streptococcus viridans Cultures

<i>Streptococcus viridans</i>			0.2 cc. antiserum for hemolytic streptococci of Groups				
Strain		Extract	A	B	C	D	E*
		cc.					
1	A 148	0.4	—	—	—	—	—
		0.1	—	—	—	—	—
2	A 84	0.4	—	±	—	—	—
		0.1	—	+	—	—	—
3	V 110 A	0.4	—	—	—	—	—
		0.1	—	—	—	—	—
4	A 49	0.4	—	—	—	—	—
		0.1	—	—	—	—	—
5	A 135	0.4	—	—	—	—	—
		0.1	—	—	—	—	—
6	B 39	0.4	—	—	—	—	—
		0.1	—	—	—	—	—
7	W 73	0.4	—	—	—	—	—
		0.1	—	—	—	—	—
8	A 141	0.4	—	—	—	—	—
		0.1	—	—	—	—	—
9	38 D	0.4	—	—	—	—	—
		0.1	—	—	—	±	—
10	K 157	0.4	—	—	—	+	—
		0.1	—	—	—	±	—

* See footnote Table I for cross-reactions of one antiserum for Group E.

When grown on the surface of blood agar plates the colonies of the last two groups (C and E) had a distinctive appearance.

After 16 hours' incubation they were translucent and many had a dewdrop or mucoid appearance. Frequently, two types of colonies were observed, one more mucoid and the other almost pin-point; this type tended to become predominant after prolonged cultivation on artificial media. Animal passage restored the



TEXT-FIG. 2. Development of gastric retention (prolongation of rate of passage associated with increased erosion of metal) under different dietary conditions in 3 rats. In Rat 797, gastric retention initiated by diet low in protein and high in fat—became fatal later with diet low in protein and high in carbohydrate. In Rat 866, gastric retention promptly produced by diet of white bread—not definitely relieved later by diet high in fat and low in protein. In Rat 909, diet low in protein and high in fat did not give rise to gastric retention but transient retention was produced by diet high in protein after period of protein restriction. For key to types of test material used and indicated in curve of erosion see Text-fig. 1.

of *Streptococcus viridans*, included as controls, did not fall into any of these groups. The strains of hemolytic streptococci were of human, bovine, and other animal origin, and also included several strains from cheese. They were obtained from numerous laboratories, chiefly in this country and Canada, with a few from England and Germany. It is an interesting phenomenon that the animal source was closely correlated with this grouping. Thus, Group A comprised chiefly strains of human origin; Group B, chiefly strains of the high acid-producing, sodium hippurate-hydrolyzing variety isolated from bovine and dairy sources; Group C contained strains from a variety of animal sources and included those strains of bovine origin which attained an intermediate final pH of about 4.8 and did not hydrolyze sodium hippurate; Group D included strains from cheese only; and Group E comprised three strains from certified milk, and may be only a small number representing a larger group. Doubtless, other groups would be found if streptococci from other sources were examined, but this series seems sufficient to establish the principle of specific group differentiation by serological methods. The subdivision of some of these groups into specific types has been accomplished by methods discussed elsewhere, and further work on this subject will be reported in another paper.

A few minor cross-reactions between groups were observed, but they were so slight as not to interfere with the specific identification of the strain in question. The results of the serological method of differentiation were brought into relationship with those based on biochemical and cultural reactions by a comparative study of the tests which, from the rather large number available, had been found most satisfactory by other investigators, together with an additional means of differentiation dependent upon marked susceptibility to streptococcus bacteriophage. The striking correlation between the results of the serological method and those based on biochemical and cultural reactions confirms the classification obtained by the serological method and adds much weight to its validity. Members of the groups differentiated by either method can usually be distinguished by their biochemical characteristics; they are, however, much more easily and specifically identified by the anti-C precipitin test.

The specificity of the precipitin reaction is probably dependent upon

restriction and undernutrition. In all cases in this study, the gastric retention associated with the beginning of protein realimentation was soon reduced or cleared up entirely with further realimentation. But similar or more acute gastric retention, when realimentation was attempted after prolonged fasting in an earlier study (10), evidently accounted for the deaths of a number of rats.

An investigation of the effect of alcohol, added to the diet, was also made in the present study. Dry food was soaked in solutions of alcohol (from 25 to 50 per cent) and offered as a paste (alcoholic diet) to the rats. Often the animals refused to partake of the fresh mixture and waited until more or less of the alcohol had evaporated. The higher concentrations evaporated so much faster than the weaker solutions that the amount which the animals took and the net effects were apparently the same. Gastric retention, following the giving of alcoholic solutions to rats, has already been observed by Cori, Villiaume and Cori (11). In the present tests, alcoholic diets low in protein produced greater gastric retention and correspondingly more erosion than diets in which alcohol was not used. With alcoholic diets adequate in protein, gastric retention was also produced or prolonged, in spite of substantial gains in the weight of some of the animals (Rat 912, Text-figs. 3 and 4). This again showed that gastric retention or its absence did not depend directly upon the general condition of the animals. Retentions produced by alcoholic diets promptly cleared up again when the use of alcohol was discontinued.

Text-fig. 4 indicates the results when a somewhat simpler method was tried than that used in obtaining the data concerning gastric retention upon which Text-figs. 1, 2 and 3 were based. The object at first was to give only a single injection of test material daily and to use only a single type of material throughout. As the larger and rougher pieces of test material appeared to be retained in the stomach more commonly than the smaller and smoother material, it was decided to use the shanks of 2-56 iron machine screws—about the largest and roughest material we ever gave to rats (Fig. 1). However, in the hope of also demonstrating a differential gastric retention of dissimilar materials, a small and smooth type of test material (stainless steel balls) was likewise given daily (excepting when a separation of material injected during different periods was desired). The results

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confirmed what had already seemed evident; namely, that sometimes mainly the larger and rougher test material is retained while at other times all types of material, including food and fluid, seem to be retained in equal degree. With the method here used, evidence of marked erosion during the periods of gastric retention was not obtained, partly, because of the impossibility of separating material given during the different periods when the same type is given throughout and, also, because both the screw shanks and the stainless steel balls resisted erosion considerably.

The curves indicating gastric retention, particularly in Text-figs. 2 and 3, show a periodicity that suggests the introduction of an artifact by the use of the test material. That is, it seems that the accumulating test material might have stimulated motor activity, with the consequence that mass movements of material through the pylorus occurred periodically and in spite of a tendency toward retention. If this were true, a reduction of the amount of test material given daily would be expected to permit a more prolonged accumulation in the stomach and this was apparently demonstrated in the latter part of Period B, Text-fig. 4. During Period B only two-fifths as much test material was given as in Period A. The retention during Period B became more prolonged but it did not become as great absolutely. A greater reduction in the amount of test material given daily might have been still better but observations on other rats that were fed diets tending to produce gastric retention showed that considerable variations in the food intake occur, even when no metal is given. This suggests that a true periodicity, at least in the degree of gastric retention, exists. In fact, a periodicity is very likely created by the factors involved in gastric retention. Retention leads to a restricted food intake and this, as the experiments with undernutrition showed, does not favor the production of gastric retention and perhaps even helps to relieve one. When the stomach empties, food likely to cause a recurrence of gastric retention might again be eaten, and so on.

DISCUSSION

There can hardly be any doubt that data such as are represented in Text-figs. 1 to 4 serve quite well to indicate when gastric retention occurs but they tell us nothing about the state of the pylorus. The

EXPERIMENTAL PRODUCTION OF PYLOROSPASM AND GASTRIC RETENTION IN RATS

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PLATES 40 AND 41

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Pylorospasm and gastric retention in man have been regarded as important factors in the genesis of peptic ulcers (1) but the factors controlling pyloric function and gastric evacuation have continued to remain more or less obscure (2). Attempts to produce pylorospasm in dogs do not seem to have met with success (1*b*). Our own study of the subject began in an attempt to determine whether the gastric acidity of rats was increased by diets low in protein, as had been found to be the case in man (3). The methods used to determine gastric acidity in man could not well be applied to rats, but it seemed possible that the degree of erosion of suitable pieces of metal, introduced into the stomach of rats, might serve as an index of gastric acidity. Such a method was already used by Spallanzani when he gave calcareous materials (pieces of coral and shells) to animals to determine the presence of acid in the stomach (4). Obviously, the degree of erosion of metal could be used as an index of gastric acidity only provided that the length of stay of the metal in the stomach remained fairly uniform. Observations made during an earlier study (5) suggested that variations in the length of stay of metal in the stomach might be indicated by changes in the rate of passage through the entire digestive tract. Hence, it was decided to give rats pieces of iron, steel or aluminum, note the degree of erosion or the percentage of weight lost by the metal and also note its rate of passage through the digestive tract. It soon became evident that, in most instances, a striking increase in erosion occurred when diets low in protein were fed, but it was usually found that the passage of metal through the digestive tract was then also prolonged. Fluoroscopic observations (checked later by direct

and, excepting for a general hypertrophy, a digestive tract in better condition was not found in any of the other rats in our colony. Of course, an adequate diet was always supplied and the amount of metal given, although large, was never beyond the animal's capacity to pass it.

That the human stomach, under some circumstances, can bear an almost unbelievable amount of mechanical insult seems indicated by a case cited by Ewald (12), in which a sailor swallowed 35 pocket knives in the course of 10 years. At death (due to perforation of the colon by 2 blades) some 30 pieces of blades together with handles were found in the stomach, but no signs of recent or earlier ulcers! Nevertheless, the theory that mechanical irritation is an important factor in the etiology of gastritis and ulceration continues to be upheld in some quarters. In support of this view, Konjetzny and Puhl, for instance, referred to the frequent occurrence of gastric and duodenal ulceration among prematurely weaned calves (13). However, such calves are primarily undernourished because they cannot very well utilize the coarse food upon which they are forced to subsist and this undernutrition, as the results of our studies indicate, evidently renders the digestive tract susceptible to ulceration. That mechanical irritation may then assist in producing lesions is supported by some evidence also obtained in our own studies but the bulk of evidence indicates that it is a negligible factor, in the genesis of peptic ulcer, in the absence of acid gastric juice. In this connection, it is of interest that Puhl, whose work in the past largely helped to bolster the theories fostered by Konjetzny (14), more recently reported the consistent production of lesions in dogs (15) by the sham-feeding (appetite gastric secretion) technique introduced by Silbermann (16).

The data obtained in this study did not serve to show whether a change in gastric acidity, sufficient to account for the development of pylorospasm and gastric retention, ever occurred. We believe nevertheless that irritation of the pyloric region by acid gastric contents was a factor but, at the same time, we feel that an increased susceptibility of the pyloric region to irritation was a more important factor. The gastric retention which often immediately followed the use of alcohol, for instance, was very likely due to the power of alcohol to precipitate the protective mucin in the stomach. Thus, the pyloric region was evidently more directly exposed to irritation by the gastric contents,

wire about 4 mm. long. 100 pieces, weighing about 1.8 gm., were used in 3 day tests. (8) Pieces of No. 16 aluminum wire about 2 mm. long. 108 pieces, weighing about 1.2 gm., were given during 3 day periods. Occasionally, 2 day tests were made with correspondingly less material. Single-day tests were also sometimes made, especially shortly before the animals were sacrificed. In such tests, 100 steel balls were generally given in 3 or 4 injections made within about 20 minutes. (9) In addition to the foregoing test materials, shanks of 2-56 (No. 2—56 threads to the inch) iron machine screws (about 2 mm. in diameter), 4 to 6 mm. long, were used for a single experiment involving a somewhat different method.

The chief observations were made on 10 adult rats—6 males and 4 females. One male and 1 female were pied (black and white) rats; the rest were albinos. Metal was given to the 10 rats daily for periods of from 47 to 249 days. The test material was injected into the esophagus by means of a brass tube fitted with a plunger, as described elsewhere (5). The animals were kept in individual cages with screen bottoms. The diets were changed from time to time, particularly in respect to their protein content. Concentrated food (without added roughage) was fed and this was given dry, excepting in tests where solutions of alcohol were added. Fluoroscopic observations were made only when the site of stasis of metal was in question. A more systematic fluoroscopic study was not made, among other reasons, because of the possibility that pylorospasm might be relieved by the irradiation (6). Three of the rats died; the rest were killed with ether. Autopsy was performed immediately after death was noted. The digestive tract was divided into two parts by severing the duodenum. The contents of the two segments were then removed by flushing with water and, when easily possible, without slitting the parts. The segments were next fixed by filling them with, and placing them in, formalin-Zenker solution. After being fixed, the segments were slit throughout and examined.

RESULTS

In the first place, various tests showed that nearly all of the erosion undergone by pieces of iron, steel or aluminum, in passing through the digestive tract of rats, occurred in the stomach and was evidently due to the HCl. Some erosion occurred in all of the rats used, but only delays in passage involving a gastric retention of metal were accompanied by a marked degree of erosion. This was strikingly illustrated in the case of Rat 916 (Text-fig. 1) in which a cecal stasis of test material involved an average of only about 0.3 per cent erosion while a subsequent gastric retention led to 52 times as much erosion—an average of 15.6 per cent among 100 pieces of aluminum; and some of these pieces were eroded about 90 per cent within 6 days.

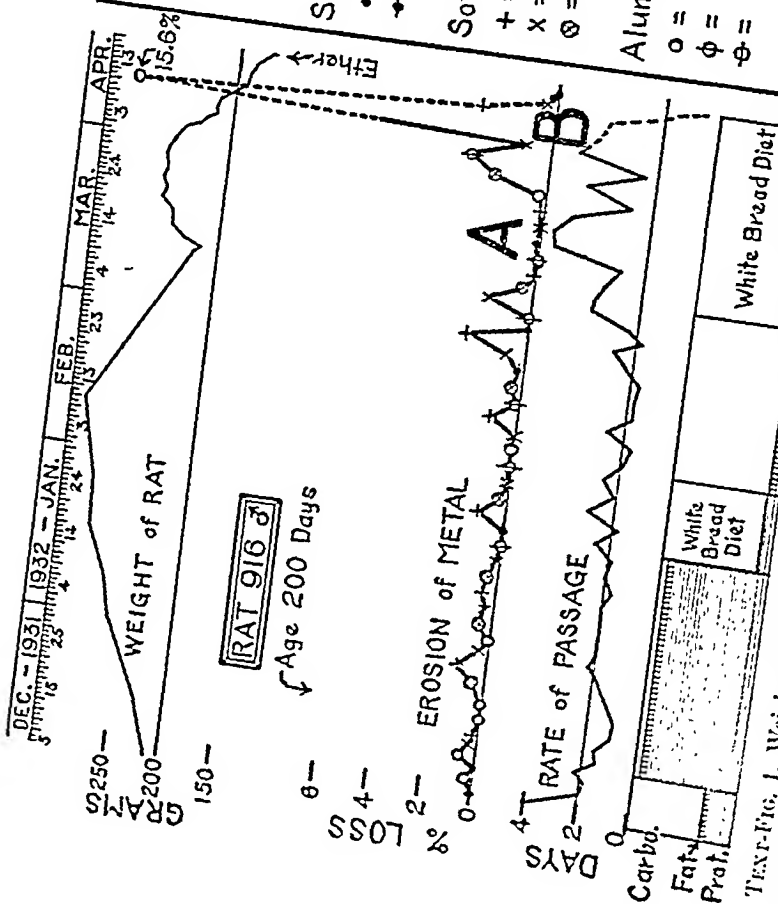
When diets low or inadequate in protein were fed, 7 out of the 10

effect of a vegetable diet (presumably grains and milk) does not apply directly to our use of diets lower in protein. Undoubtedly further study is here needed.

Diametrically opposed to the view that a deficiency of bile might account for an increase in the sensibility of the pyloric region and thus lead to pylorospasm and gastric retention is the theory propounded by Spira; namely, that bile is the cause of pylorospasm, as well as of gastric and duodenal ulceration (22). According to Spira, fat in the diet, by giving rise to biliary regurgitation, is mainly responsible for ulceration. Experiments are said to be under way to prove this theory but positive results are not likely to be secured if the observations made in our studies have significance. We fed rats various diets containing large proportions of fat (23). We could agree with Spira that fat, and particularly oil, may have no protective value against ulceration when the protein content of the diet is inadequate. But with diets adequate in protein, our results showed that fat in the diet tended to help heal, rather than produce, ulcers. Possibly Spira's therapeutic claims for diets low in fat can be explained by the protein-sparing effect of such diets.

The results of our studies appear to justify the procedure followed at Bier's clinic, where a preparation consisting mainly of bile acids is used in gastritis and ulcer therapy (24). Nevertheless, the possibility remains that bile may be highly toxic to the gastric and duodenal mucosa under some conditions, although protective under other conditions. Perhaps further work like that referred to above (17) and that of Draper and Touraine (who find constitutional peculiarities in ulcer patients) (25), McClure and Huntsinger (who found hepatic dysfunction associated with duodenal ulcer) (26) and Tashiro and his assistants (who regard bile salt retention in the blood as responsible for ulcer) (27) will eventually settle this question.

Independent of the precise rôle of bile, however, it seems of interest that, although carbohydrates tend to leave the stomach rapidly and protein more slowly in brief tests (28), the reverse proved to be the case in our more prolonged experiments. Also, diets high in fat and low in protein, which ordinarily remain longer in the stomach than diets high in carbohydrate, did not lead to gastric retention quite as readily as the latter. Furthermore, although coarse particles often



TEXT-FIG. 1. Weight of Rat 916, erosion of metal (per cent of weight lost by metal) in passing through digestive tract, rate of passage and diets used during different periods. At A, only slight erosion during period of marked cecal stasis; at B, striking increase in erosion with gastric retention. Discontinuous lines connecting points at ends of curves of erosion and rate of passage indicate that part of the metal given during that period was still in the animal at death. Note that gastric retention (at B) involves a relatively slow delayed passage of metal associated with increased erosion and occurs with diet inactivation.

crater in the antrum. The lack of more ulceration in the stomach of this rat is, in part, consistent with the fact that gastric retention never became very great in this case without a "spontaneous remission." Excepting for a little fluid (probably water), the stomach was entirely empty at the time this animal was sacrificed. That more severe ulceration did not develop in this rat, which was given the largest amount of the roughest pieces of metal for the longest time in this study, again demonstrates that mechanical irritation is not an important factor in ulcer production in rats. Rat 916 (Text-fig. 1 and Fig. 4) had a marked epithelial overgrowth in the prostomach and a number of small bleeding ulcers or craters in the antrum. The bleeding prepyloric lesions in this rat, after a prolonged low protein diet poor in fat, contrast with the relatively inactive prepyloric craters in Rats 797 and 912 above. Rat 916 (Fig. 4, *N*) and two other rats in this study also had one or more nodules apparent on the outer or inner surface of the prostomach. Such nodules were previously found to be consequences of ulceration in the prostomach of rats (10).

In earlier studies in which rats were not given pieces of metal but were fed inadequate diets such as were used in this study, gastric ulceration, epithelial overgrowth and nodule formation also occurred (8, 10, 23). In fact, more marked lesions than any seen in the present study were not uncommon. This further indicates that the lesions found in this study were mainly consequences of nutritional disturbances and not of irritation by the test material employed.

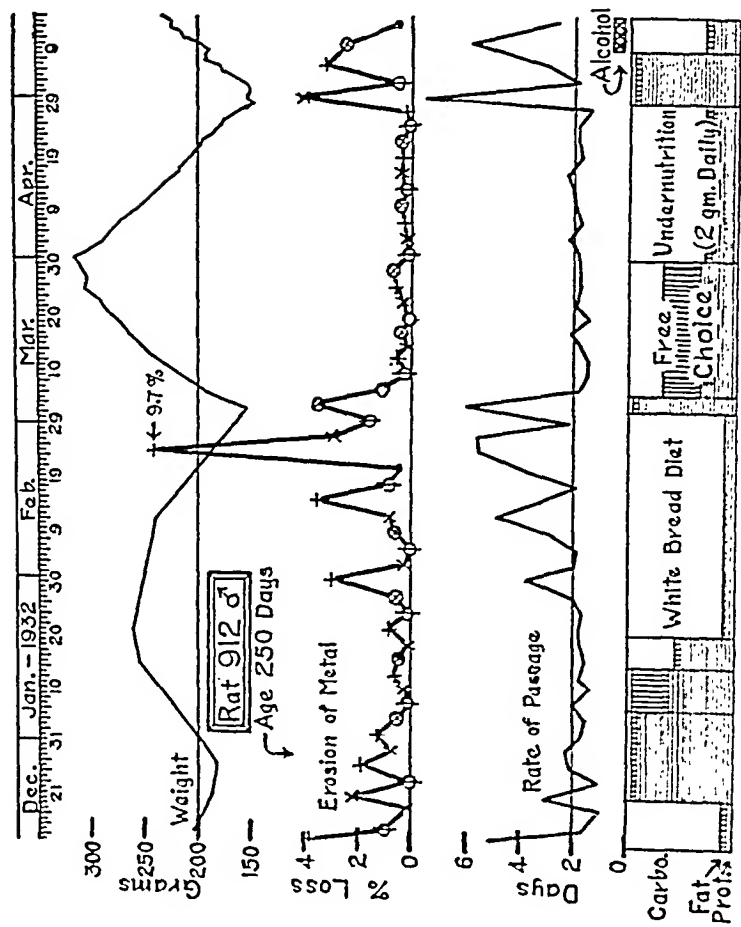
To what extent the findings on rats are transferable to man has become something of a question in view of our observation that differences already exist between rats and mice. However, both rats and mice are omnivores like man; both species develop lesions with inadequate diets and it is our belief that further studies on these and other omnivores would best serve to bring to light those factors in the development of peptic ulcer that apply alike to the lower animals and to man.

SUMMARY

1. A method was found whereby the development of gastric retention could be studied in intact animals without the necessity of recourse to the use of x-rays.

2. Gastric retention was found to develop as a result of protein restriction in 7 out of 10 rats studied. Such retention could again be cleared up with protein realimentation or by allowing the animals free choice of protein, fat and carbohydrate.

3. A diet high in protein following periods of undernutrition or



TEXT-FIG. 3. At the beginning is shown moderate gastric retention with diet low in protein; next, decreasing retention with diets high in protein; then increasing retention with diet of white bread, followed by transient prolongation of retention with diet high in protein; next, absence of gastric retention with food permitting free choice of protein, carbohydrate and fat and no retention with marked undernutrition, using diet with 21 per cent protein; then transient retention with diet high in protein, and, finally, gastric retention with alcohol added to diet. For key to test material used see Text-fig. 1.

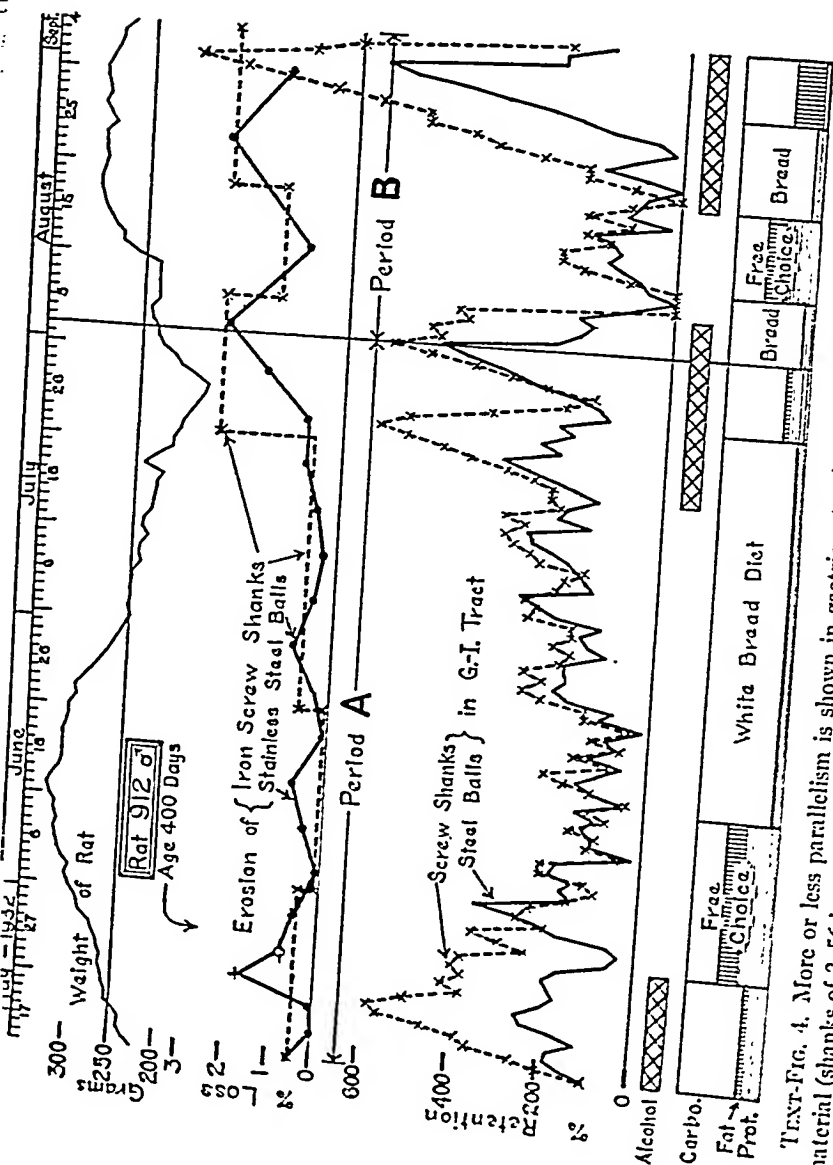
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EXPLANATION OF PLATES

PLATE 40

FIG. 1. Photograph and roentgenogram of test material. Groups A and B used in intestinal rate study; Group A used on rats; Group B, on larger animals (5). Groups C and D used for mechanical irritation of digestive tract. Part of Groups A, C and D used in the present study. Group A (from left to right)—glass beads, glass balls, gold balls, silver balls and 1/16 inch steel balls (used in this study). Group B—knots made of colored cotton thread, glass beads, glass balls, steel ball (above) and gold ball (below), marked gold discs and marked silver discs. Group C—steel, iron, aluminum, silver and gold wire; at left, round wire; at right, square wire. Of this group, used in present study, No. 15 soft iron wire (fourth from left); No. 18 aluminum wire (fifth from left), and No. 16 aluminum wire (sixth from left). Group D—gold, silver, aluminum and iron test material; at left, corrugated; at right, threaded material. Used in this study, shanks of 2-56 iron machine screws (fifth row from right). Scale in centimeters and inches.

FIG. 2. A, roentgenogram of Rat 15, during period when it was given 300 pieces of metal daily, showing 182 pieces of threaded and corrugated silver wire in digestive tract. S, about 125 pieces of metal in stomach (150 pieces were given earlier in day); Ce, mass in cecum; Co, large pellet with metal in colon; scattered single pieces are in small intestine. B, photograph, and C, roentgenogram of 24 hour collection of fecal pellets from Rat 15 during same period, showing distribution of metal (305 pieces) in pellets.



TEXT-FIG. 4. More or less parallelism is shown in gastric retention of a large and rough type of test material (shanks of 2-56 iron machine screws) and a small and smooth type of material (1/16 inch stainless steel balls). During Period A, we gave 5 shanks of 2-56 screws (about 0.6 gm.) and 40 steel balls (about 0.6 gm.) daily, excepting as noted in curve of erosion of steel balls (key for exceptions with Text-fig. 1). During Period B, gave two-fifths as much test material as in Period A. Per cent retention means per cent of amount of test material given daily and retained more than 24 hours. Gastric retention produced mainly by 25 per cent solution of alcohol added to type of diet indicated.

presence of more or less pylorospasm during all periods of gastric retention in this study was nevertheless inferred from the findings at autopsy. In the first place, although the stomach was usually large, there was nothing to indicate that an atonic condition, such as might account for the retention, ever existed. In the second place, the pylorus of rats with gastric retention generally appeared to be markedly constricted (Fig. 4), and finally, when the digestive tract of such rats was filled with fixing fluid, shortly after death, the pylorus acted as if it were spastic. In our various gastrointestinal studies, we have filled the intact stomach of a large number of rats with formalin-Zenker solution, by injecting it either through the esophagus or through the duodenum. In rats that had been kept on diets that tended to produce gastric retention, the passage of the fixing fluid was often obstructed entirely at the pylorus, while in rats that had not been kept on such diets, the fluid usually passed the pylorus easily. A pyloric hypertrophy, such as sometimes occurs in human infants, was, however, never seen; nor did we ever note anything suggesting cardiospasm in a rat.

Granting then that pylorospasm was the cause of the gastric retention of metal, it remains to explain the onset of pylorospasm. Simple mechanical irritation of the pyloric region by the test material can hardly be responsible, since, in spite of continuing to give the same amounts and kinds of test material daily, it repeatedly proved possible to clear up gastric retentions entirely by making simple changes in the diets. The difficulty of producing gastrointestinal lesions by mechanical irritation has already been referred to in a previous report (5), but this might be emphasized by citing the following case from a further study.

Rat 15 (Fig. 2) was given over 50,000 pieces of gold and silver—a total of over 10 times the animal's average weight—during a period of 557 days. Beginning at the age of 21 days, the amount of metal given daily was increased from time to time until 300 pieces of silver were given daily during the last 15 days. About 90 per cent of the metal given to this rat consisted of round and square wire and over half of this was either threaded or corrugated (Figs. 1 and 2). In spite of this, Rat 15 grew to be the largest female among a colony of about 1500 rats—weighing 432 gm. net (minus metal in tract) at the time it was sacrificed. Although kept separated from males part of the time, it also had 5 litters and raised most of the young, while receiving metal daily. Gastric retention apparently never developed

with the result that pylorospasm promptly developed. Likewise, the gastric retention produced by protein restriction and the transient retention following periods of undernutrition may have been due to a decrease in the secretion of mucin, but we are more inclined to believe that a change in the character and/or quantity of bile secreted was the most important factor involved here.

The importance of biliary or hepatic factors in the development of duodenal and gastric lesions has been emphasized by the work of Kapsinow and others (17). These investigators showed that duodenal ulcers develop in dogs when the biliary secretion is diverted so as not to reach this region. In contrast to this, rats apparently furnish an illustration of a species in which duodenal ulceration is largely prevented by a more or less continuous flow of bile into the duodenum. In our studies on about 1500 rats, we neither found nor were able to produce duodenal ulceration in a single case, although severe erosion sometimes occurred. (Bullock and Rohdenburg published gross illustrations (but not sections) of "spontaneous" duodenal ulcers in two rats (18) but we never saw anything like this.) On the other hand, in the course of a preliminary investigation on mice, we found or produced duodenal lesions in at least 5 out of 60 animals. Mice differ from rats in having a gall bladder, while rats, without a gall bladder, apparently have a more continuous flow of bile into the duodenum (19). If, then, Whipple's conclusion concerning the relation of protein metabolism to bile secretion (20) is applicable to rats and mice, protein restriction might be expected to have reduced the bile flow in rats only enough to permit an irritable pyloric condition and prepyloric lesions to develop but not duodenal ulcers, while inadequate diets and undernutrition in some mice permitted the development of duodenal lesions also. Again, applying Whipple's conclusions, the transient gastric retention that generally developed in rats when a diet high in protein was used after a period of undernutrition would be due to the lag in the increase of bile flow that occurs with protein realimentation. In apparent conflict with this explanation is McMaster's failure to observe any difference between the effect of a vegetable diet and a meat diet on the secretion of bile in rats (21). However, assuming that no error was introduced by the special technique used by McMaster in collecting bile, his observation on the

remain in the stomach longer than finely divided material, it has long been known that coarse (Graham) bread leaves the stomach faster than white bread (29). Our work confirmed this by showing no definite gastric retention when diets consisting mainly of bran or other roughage were used while diets of white bread led to the most acute retention. Taken together, these results make it obvious that some factors of importance in pyloric function and gastric evacuation have not been taken into consideration in earlier studies.

As pylorospasm and gastric retention have been regarded as important factors in the genesis of peptic ulcers in man, it seemed of interest to note whether such a relationship also existed in the rats. The appearance of the normal stomach of the rat is indicated in Fig. 3 and the gastric conditions and types of lesions found in the present study are illustrated in Fig. 4. All rats, including those that never developed a gastric retention in this study, showed evidence that more or less ulceration occurred in the prostomach at some time. This and earlier observations indicate that, although gastric retention may intensify ulceration in the prostomach, ulceration in this region can occur independently of retention. Seven of the 10 rats showed some crater or ulcer formation in the prepyloric region. The exceptions were the only 2 rats that never developed gastric retention in this study and 1 rat (Rat 911) that developed retention while fed a diet of white bread and was then relieved of the retention with an adequate diet (which might also have led to the healing of prepyloric lesions) and finally died while fed only small amounts of food, without retention. These results suggest that a definite relation existed between the occurrence of gastric retention and the presence or production of prepyloric lesions but the precise relation can only be determined by further study.

Rat 797 (Text-fig. 2 and Fig. 4), which was the only rat in this group that died with a marked retention of fluid in the stomach, developed the most severe ulceration in the prostomach. A number of small shallow craters or beginning ulcers appeared in the antrum. Craters or ulcers usually develop much more slowly in the antrum than in the prostomach of rats; and a high fat, low protein diet, such as was used to initiate gastric retention in this rat, generally did not give rise to as marked crater formation or ulceration in the prepyloric region as low protein diets with a high carbohydrate (starch) content. Rat 912 (Text-figs. 3 and 4 and Fig. 4) had only one small ulcer in its prostomach and only one small (non-bleeding)

of the results of skin tests with Type I S.S.S. obtained in a series of 53 cases of Type I pneumococcus pneumonia, 48 of which were treated with antipneumococcus Type I serum.

Materials and Methods

The patients were among those admitted to the wards of the Hospital of The Rockefeller Institute with a diagnosis of lobar pneumonia. Their ages ranged from 6 years to 70 years. The pneumococcus typings were done by use of the Avery tube, as well as by the usual method of inoculating the sputum of the patient into the peritoneal cavity of a mouse, and the subsequent macroscopic agglutination and precipitation reactions with peritoneal washings of the mouse. Blood cultures were made on admission and at varying intervals thereafter. Except in the 5 instances noted, all patients were treated with unconcentrated Type I antipneumococcus horse serum obtained from the New York State Board of Health. Specific treatments consisted in the intravenous administration every 6 to 8 hours of 100 cc. of serum diluted with an equal volume of physiological saline.

Skin Test.—Protein-free type-specific capsular polysaccharides were prepared by the methods employed in this laboratory (4) and were of the highest degree of purity obtainable. The polysaccharides were dissolved in physiological salt solution, and 0.1 cc. of solution containing 0.01 mg. of the S. S. S. was injected intradermally. In the earlier tests, S. S. S. of Types I, II, and III were separately injected into the skin of the forearm together with a saline control. In the present study of reactions in patients with Type I pneumonia, only the Type I S. S. S. and a saline control have been used.

The skin tests, during the course of treatment, were done 6 to 8 hours following each administration of serum, since it is known that after this interval the passively introduced antibodies may have been removed from the blood (5). Following each intradermal test, the inoculated sites were observed carefully for 20 to 30 minutes. A positive reaction was recorded in the presence of erythema, edema, wheal, and pseudopodia. Frequently, itching of the skin at the site of reaction was noted by the patient. Concomitantly, the elevation of the skin at the point of the control saline injection tends to disappear.

Circulating Antibodies.—The presence of type-specific agglutinins for Type I Pneumococcus was demonstrated in the patient's serum by the method previously described (2).

The data included in this report comprise the results obtained by repeatedly testing the skin reaction to Type I S.S.S. in 53 patients during the course of lobar pneumonia associated with Type I Pneumococcus. In 48 of the cases specific serum therapy was employed. In the series are included 7 fatal cases, 5 cases with empyema, and others with furuncles, sterile pleural effusions, and delayed resolution.

prolonged protein restriction usually gave rise to a transient gastric retention.

4. Diets with a solution of 25 per cent or more of alcohol added promptly gave rise to gastric retention in rats even when the protein content of the diet was adequate.

5. Evidence is given indicating that the gastric retention which occurred in this study involved more or less pylorospasm and the possible influence of mechanical and chemical irritation and of changes in gastric mucin and bile flow upon the development of pylorospasm are discussed.

6. Spira's theory that fat in the diet gives rise to pylorospasm and ulceration is not supported by the results of our experiments.

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zation of the blood, the presence of circulating antibodies, and symptomatic improvement, the skin reaction remained negative. The fact that the skin test, under these conditions, is negative, clearly shows

Key to Charts 1 to 6

Skin Tests.—Broken line = negative reaction. Wavy line = doubtful reaction. Cross-hatched bar = positive reaction.

Agglutinins.—Dash = no demonstrable agglutinins. Black bar = highest positive dilution.

† Indicates fatal termination.

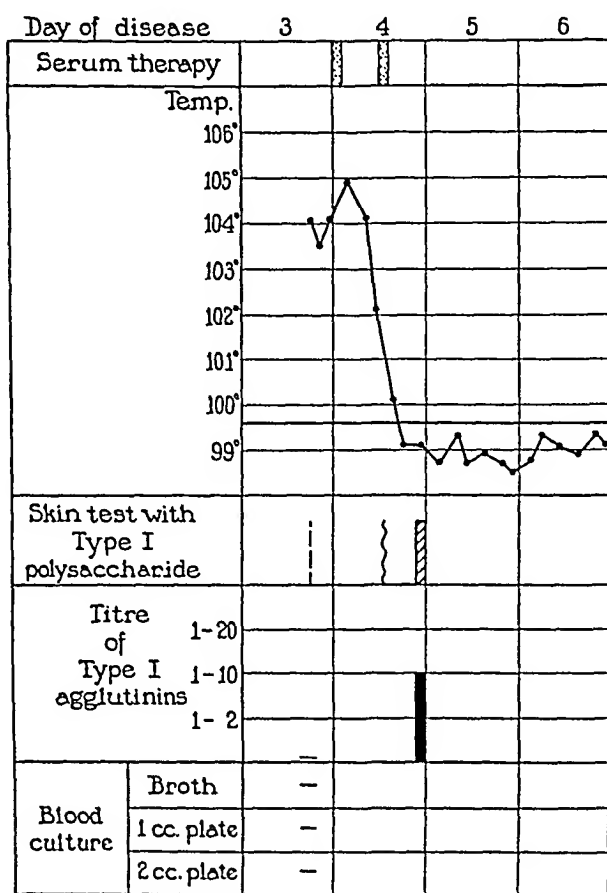


CHART 1. Patient S (Hospital No. 7152). Type I pneumococcus pneumonia without septicemia.

that positive reactions are not simply dependent upon the humoral type-specific antibodies, and that cutaneous reactivity is not solely referable to the passive immunity conferred by the administration of the specific antibodies

PLATE 41

FIG. 3. Appearance of normal stomach of rat. At left, ventral or anterior half of an empty stomach (Rat 700); at right, dorsal or posterior half of a full stomach (Rat X-228). *Es*, esophagus; *PS*, prostomach—lined with squamous epithelium like esophagus; *R*, ridge or fold between prostomach and main stomach; *MS*, main stomach or ventriculus—acid-secreting part of stomach; *An*, antrum or prepyloric region, and *Py*, Pylorus. Rat 700 weighed 200 gm. when sacrificed and had been kept on a high fat (30 to 45 per cent) diet, with cut up dog hair included, during the preceding 4 months. Rat X-228 weighed 195 gm. when sacrificed and had previously been on a stock diet of meat, milk, bread, cracked corn and greens.

FIG. 4. Gastric conditions and types of lesions found in present study; Rat 797 (above), Rat 912 (middle), and Rat 916 (below). Pylorus constricted and stomach enlarged in each case. *C*, sites of small craters or ulcers in prepyloric regions (both halves of stomach of Rat 916 with prepyloric lesions). *U* (Rat 912), ulcer in prostomach at ridge. *N* (Rat 916), nodule or small tumor-like prominence evident on outer surface of prostomach.

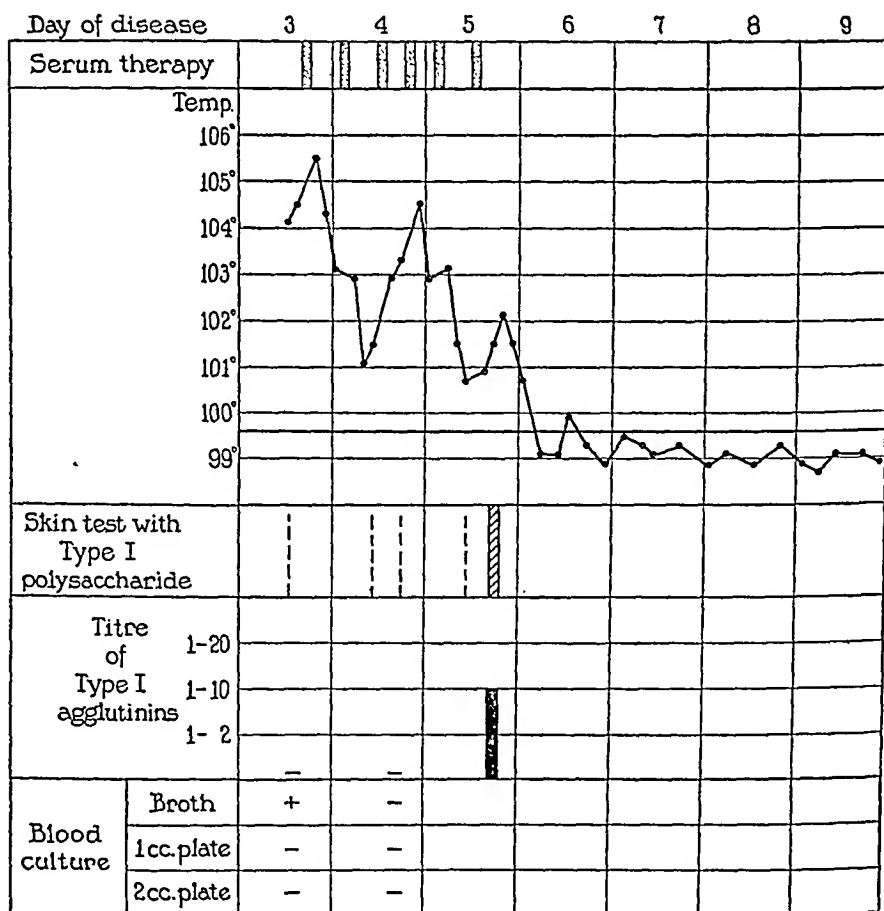


CHART 2. Patient P. (Hospital No. 7263). Type I pneumococcus pneumonia and septicemia.

Patient 3, Chart 3, Case 7165

The patient, a housewife of 30 years, was admitted to the hospital on the 2nd day of illness, suffering from lobar pneumonia of the left lower lobe. At the time of admission, the blood culture was positive for Type I *Pneumococcus*. The patient's serum contained no demonstrable type-specific antibodies, and the Type I skin test was negative. After two doses of serum, the blood became sterile, type-specific antibodies were present in the blood, but the skin test remained negative. The pneumonic process spread to involve the left upper lobe. Serum was repeatedly administered, agglutinins persisted in the blood, but repeated skin tests were consistently negative. On the 6th day, after nine doses of serum, although no change in the patient's general condition was noted, a positive skin reaction was elicited. This was construed as indicative of recovery. The titre of circulating antibodies had not increased above that found on the 3rd and 4th

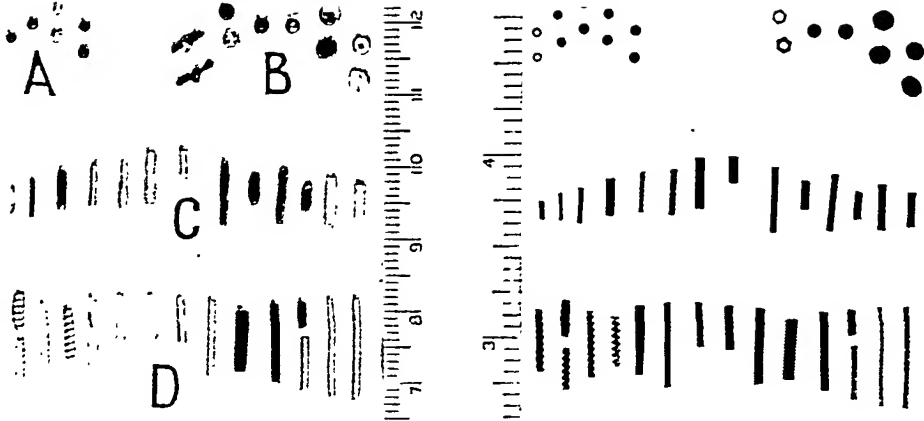


FIG. 1

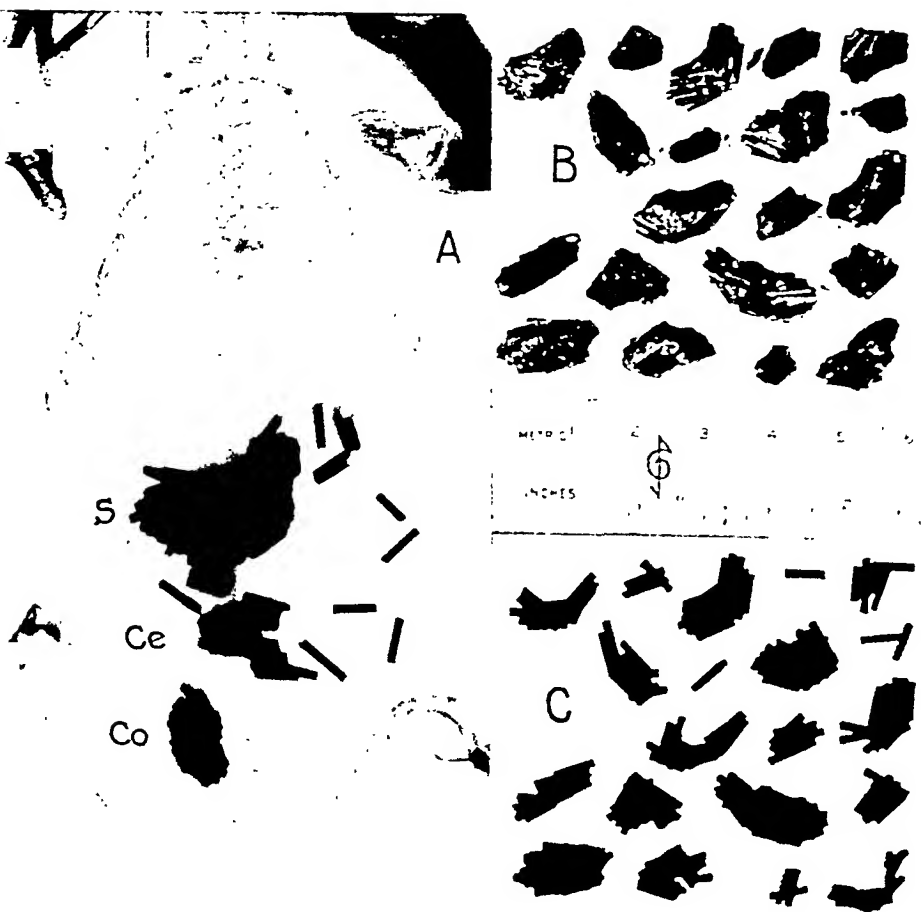


FIG. 2

Revised by the Department of the Interior

simply to the presence of circulating antibodies, but is associated definitely with the complicated process of recovery.

Patient 4, Chart 4, Case 7219

The patient, an advertising man of 52 years, was admitted to the hospital on the 6th day of disease, suffering from lobar pneumonia of the left lower lobe, left upper

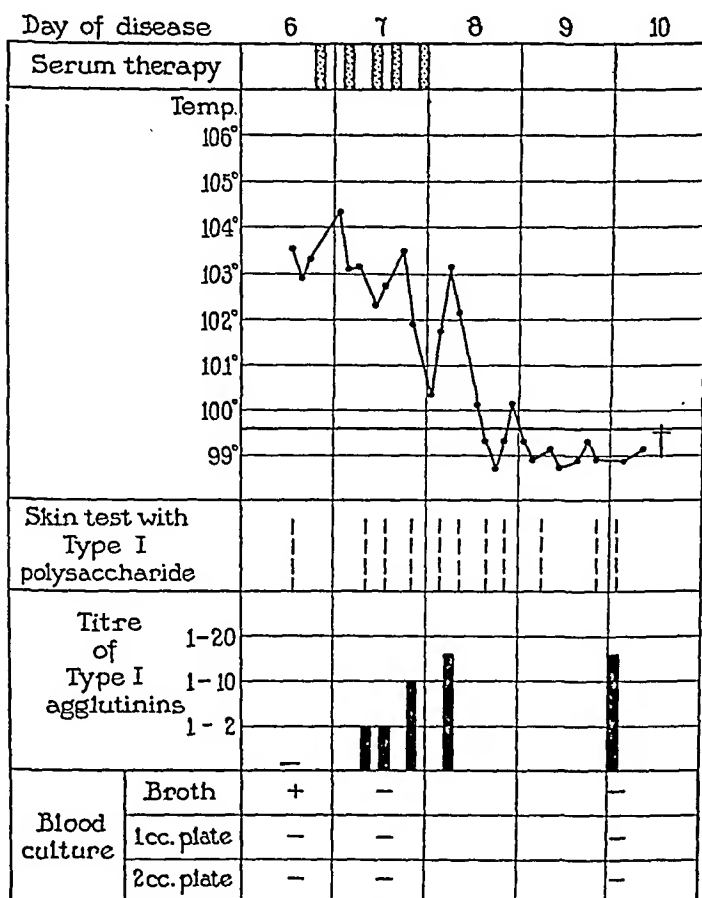
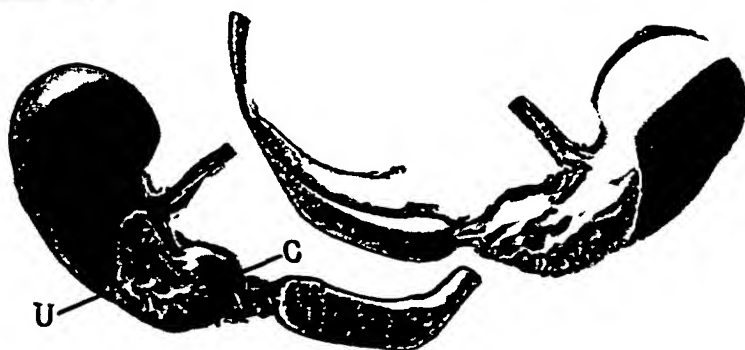
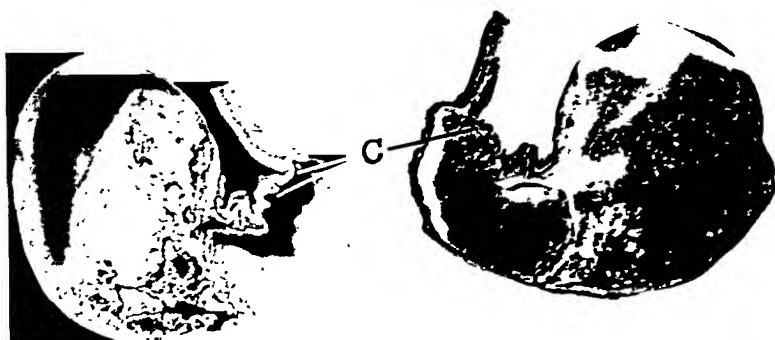
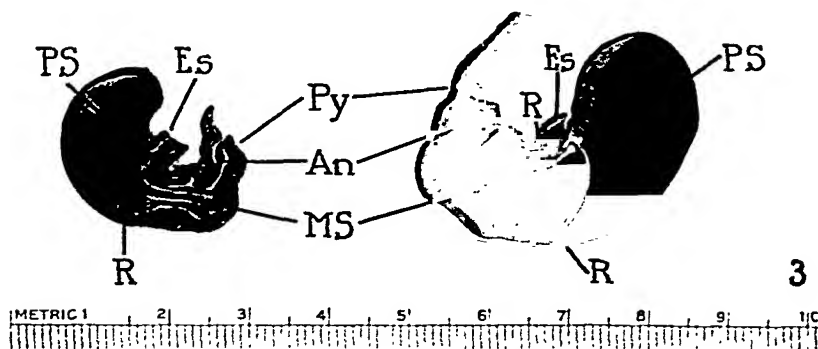


CHART 4. Patient Sc. (Hospital No. 7219). Type I pneumococcus pneumonia and septicemia.

lobe, and right lower lobe. He was extremely sick. The blood culture on admission yielded Type I Pneumococcus. There were no type-specific antibodies in the blood. The skin reaction was negative. Serum therapy was inaugurated 2 hours after admission, and continued at 6 hour intervals for five doses. After the second dose, agglutinins were demonstrated in the blood, and these increased progres-



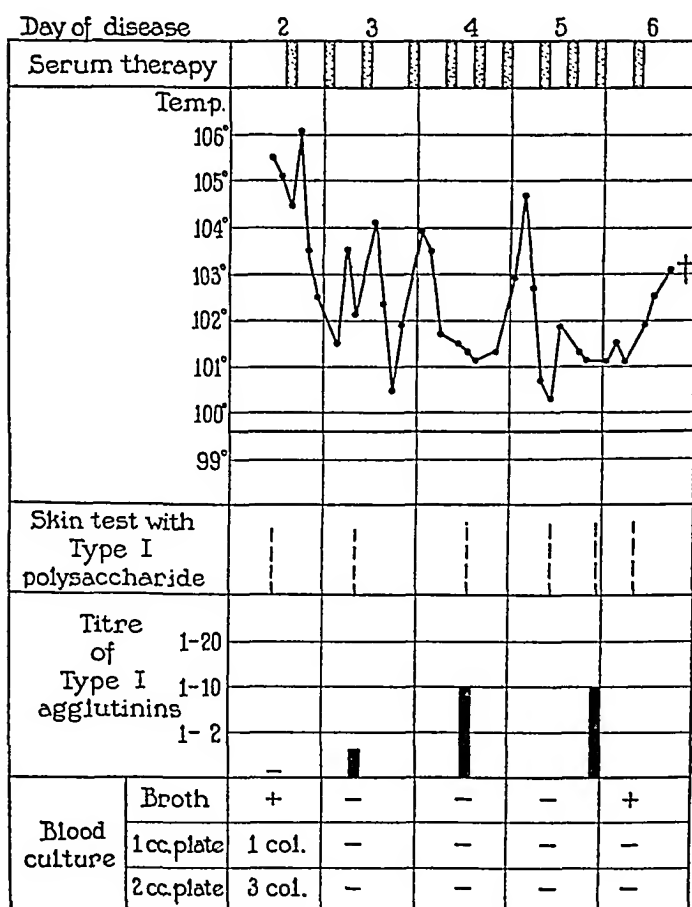


CHART 5. Patient Mo. (Hospital No. 7162). Type I pneumococcus pneumonia and septicemia.

Patient 6, Chart 6, Case 7580

The patient, a negro of 35, was admitted to the hospital on the 6th day of disease, with lobar pneumonia of the right lower lobe, and left lower and upper lobes. He was extremely sick. The blood culture was positive, no circulating Type I agglutinins were detectable, the skin test was negative. Serum therapy was instituted 8 hours after admission. After the second treatment, the temperature was lower, the blood culture in broth was sterile, no circulating agglutinins were demonstrable, and the skin test remained negative. On the 8th day, after the fifth and sixth treatments, skin tests were still negative, but a high titre of agglutinins was present, and although the temperature was normal, the patient remained dangerously ill, in delirium, and the septicemia had increased in degree. Although it was not known until later that the septicemia had increased, serum therapy was continued because of the negative skin test. On the 9th day the temperature re-

THE VALUE OF THE SKIN TEST WITH TYPE-SPECIFIC CAPSULAR POLYSACCHARIDE IN THE SERUM TREATMENT OF TYPE I PNEUMOCOCCUS PNEUMONIA*

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It has previously been shown that in patients convalescent from pneumococcus lobar pneumonia the intradermal injection of type-specific capsular polysaccharide (S.S.S.) elicits a typical cutaneous reaction (1, 2). The reaction takes the form of an immediate wheal and erythema which reaches its height within 15 to 30 minutes. It can first be elicited at or about the time of recovery. The reaction is type-specific in that it is produced only by the polysaccharide homologous in type to that of the infecting organism. Furthermore, in the small series previously reported, it was noted that recovery from Type I pneumonia in serum-treated cases was invariably associated with the development of a positive skin reactivity to the Type I S.S.S., whereas approximately only 50 per cent of the non-serum-treated Type II and Type III cases which recovered gave a specific response to the homologous S.S.S. (2). The occurrence of the reaction was found to be intimately associated with the presence of type-specific antibodies in the patient's serum. In cases treated with Type I antipneumococcus serum, it was observed that although type-specific antibodies might be present in the blood of the patients, the skin test remained negative. When, however, the skin test became positive, recovery invariably ensued.

These observations suggested that in patients suffering from Type I pneumococcus pneumonia the skin test with Type I S.S.S. might be a valuable guide in determining when serum therapy could be safely discontinued. The present report, therefore, comprises an analysis

* Presented, in part, before the American Society for Clinical Investigation at a meeting held in Atlantic City, May 4, 1931.

is possible that in the skin of a negro the milder forms of reaction are more difficult to detect. At any rate, on the 8th day, 18 hours before it was known that septicemia had increased, with the temperature normal and type-specific agglutinins present in the blood, the value of the negative skin test in indicating further treatment must be stressed. This case is also exceptional in that it remains the only 1 of the 5 cases of empyema, all of which recovered, that failed to give a positive reaction at the termination of the phase of acute pneumonia.

DISCUSSION

The results of the skin tests with Type I S.S.S. in 53 cases of Type I pneumococcus pneumonia are strikingly uniform for a biological test. In the series there were 7 deaths, in all of which repeated skin tests were negative. On the other hand, in 46 recovered cases, including 5 cases of empyema, all but 1 gave a positive reaction at about the time of recovery from the acute pulmonary process. In the one exception, a negro with subsequent empyema, only a doubtful reaction was obtained although the patient recovered (Patient 6, Chart 6). Exemplary individual cases have been cited to show the relation of positive reactions to recovery and to the presence of antibodies in the circulating blood. Furthermore, they emphasize the fact that the mere presence of circulating antibodies, in cases before recovery or in cases terminating fatally, is not sufficient to bring about a positive skin reaction.

Finland and Sutliff, in similar studies, reported only 7 positive tests to the Type I S.S.S. in a series of 15 non-serum-treated patients who recovered from Type I pneumonia (3). Of 17 Type I and Type II cases tested before the time of crisis, 3 gave positive tests to the homologous polysaccharide. Moreover, the relation of positive skin tests to recovery and to the presence of type-specific antibodies in the blood was corroborated. Of the fatal cases in their untreated series, only 1 of 11 cases of all three types gave a positive reaction. The details of this case are not recorded.

In their cases which were treated with serum (6), two distinct differences from the procedures employed by us must be noted. In the first place, Felton's concentrated serum containing Type I and Type II antibodies was used. Secondly, the skin tests were frequently

The 5 untreated patients were comparatively mild cases which recovered spontaneously. A summary of the cutaneous reactions of Type I S.S.S. in these 53 patients is presented in Table I.

The uniformity of results is surprisingly sharp for a biological test. From Table I it is readily seen that of the 45 cases in which a positive skin reaction was obtained, all recovered. Of the 8 patients with negative reactions, only 1 survived. This exception, the failure of a recovered patient to react, occurred in a negro with empyema, in whom only a doubtful reaction was seen. Further, of the other 4 cases of empyema tested after the acute stage of the pneumonia had passed, all gave typical positive reactions, and all recovered.

TABLE I

The Results of Skin Tests with Type I Polysaccharide in Type I Pneumococcus Pneumonia

Patients	No. of cases	Positive reactions		Negative reactions	
		Recovered	Died	Recovered	Died
Serum-treated.....	48	40	0	1*	7
Untreated.....	5	5	0	—	—
Total.....	53	45	0	1	7
Empyema†.....	5	4	—	1	—

* Empyema with doubtful reaction.

† Included also under serum-treated cases.

Included amongst the 7 fatal cases is 1 case which had a Type I pneumonia with septicemia, from which recovery apparently ensued with normal temperature, sterile blood, and definite and nearly complete resolution. The skin test remained negative, and the patient suddenly had a recurrence of pneumonia and septicemia, this time a Type III pneumococcus infection, which proved fatal.

A brief summary and graphic representation of illustrative cases serve to emphasize the time relationships between the onset of recovery and the occurrence of the skin reactions, with reference to the presence of circulating antibodies in the blood of convalescent cases. Furthermore, in the fatal cases, it will be seen that in spite of sterili-

The value of the skin test as employed in the present group of cases appears to be definitely established both as a guide to serum therapy and as a prognostic aid. In certain instances, by the time a typing of the invading organism is obtained, the patient appears improved and the temperature may have approached a normal level. There may be some doubt as to whether treatment with serum is required. A skin test with the S.S.S. may be done, and the result read in 15 to 30 minutes; a negative test indicates serum therapy. In all cases of this sort, the conclusions reached as a result of the skin test have been validated by subsequent findings and the course of disease. Similarly, in cases with intermittent fever, or in which a drop of temperature follows serum therapy, the skin test gives a distinct answer to the problem of whether recovery has begun or whether treatment should be continued (Charts 1 to 3). A positive reaction is invariably, in our experience, a sign that recovery will follow. Furthermore, although the patient may still appear quite sick with little change from a previous period when the skin test was negative, the development of a positive cutaneous reaction indicates recovery and that further serum therapy is unnecessary (Chart 3). A persistently negative reaction, even in the presence of circulating antibodies, clinical improvement, and continued serum therapy, offers an unfavorable prognosis, and in all cases has been associated with a fatal termination (Charts 4 and 5). To repeat, a positive immediate wheal and erythema reaction to Type I S.S.S. in cases of Type I pneumococcus pneumonia predicates recovery; a negative test calls for further serum therapy.

It has been assumed that the chief function of antipneumococcus serum as a therapeutic agent is to furnish type-specific antibodies. Consequently, the presence or absence of type-specific agglutinins in the patient's blood has been employed as a means of estimating when sufficient serum has been administered. The application of titration of circulating agglutinins in conjunction with the use of concentrated serum has been recently advanced by Sabin (7). From the point of view of recovery, however, it furnishes an estimate of only the humoral aspect. In Patient 3, Chart 3, type-specific agglutinins were demonstrable in the patient's blood although the pneumonic lesion was spreading. Later when the skin test became positive, the agglutinins were present in the same concentration. In Charts 4 and 5 are shown

Patient 1, Chart 1, Case 7152

The patient, a housewife of 32 years, was admitted to the hospital on the 3rd day of illness, with lobar pneumonia of the right middle lobe. On admission, the blood culture was negative, the blood contained no agglutinins for Type I Pneumococcus, and the skin test with Type I S. S. S. was negative. Type I antipneumococcus serum was first administered 10 hours after admission. 8 hours later, Type I S. S. S. elicited only a questionable skin reaction, since an erythema also occurred at the site of the control saline injection. Although the temperature had begun to fall, a second treatment was given, and a precipitate drop of temperature took place. A skin test done 8 hours after the last serum treatment was markedly positive, and the patient's serum at this time contained a comparatively high titre of type-specific antibodies. Convalescence was uneventful.

This case illustrates the ordinary rapid termination of the acute course of disease, and the development of cutaneous reactivity at the time of recovery.

Patient 2, Chart 2, Case 7263

The patient, a man, 34 years of age, was admitted to the hospital on the 3rd day of illness, with lobar pneumonia of the right lower lobe. On admission, a blood culture revealed Type I Pneumococcus in the circulating blood; the patient's serum contained no demonstrable type-specific antibodies; and the type-specific skin test was negative. Serum therapy was begun 6 hours after admission. After the second treatment, a fall of temperature occurred. A skin test done at this time was negative. The temperature rose again just before the fourth treatment. The skin test was negative, a blood culture was sterile, and type-specific agglutinins were not demonstrable in the patient's blood. A skin test before the sixth treatment was negative, but 6 hours later a definitely positive skin reaction was elicited with S. S. S. I, and circulating antibodies were present. Although the patient's temperature was 102°, serum therapy was discontinued. The following morning the temperature was normal, and an uninterrupted recovery ensued.

This case illustrates the value of the skin test in instances in which the temperature is swinging, and in which it is important to know whether recovery has begun or whether the fall of temperature is only a remission. The negative skin test indicates further serum therapy. Furthermore, when the positive reaction was obtained, in spite of the fact that fever was still present, it was shortly followed by a normal temperature. This case also emphasizes the fact that the antibodies passively introduced through serum treatment may not be detectable in the circulating blood 6 to 8 hours after the treatment, or even after several treatments.

days, when the skin test was negative. Nevertheless, serum was discontinued, and 48 hours later, on the 8th day, the temperature dropped to normal. Convalescence was uneventful.

The significant points exemplified by this case are that although type-specific antibodies were present in the patient's blood after two

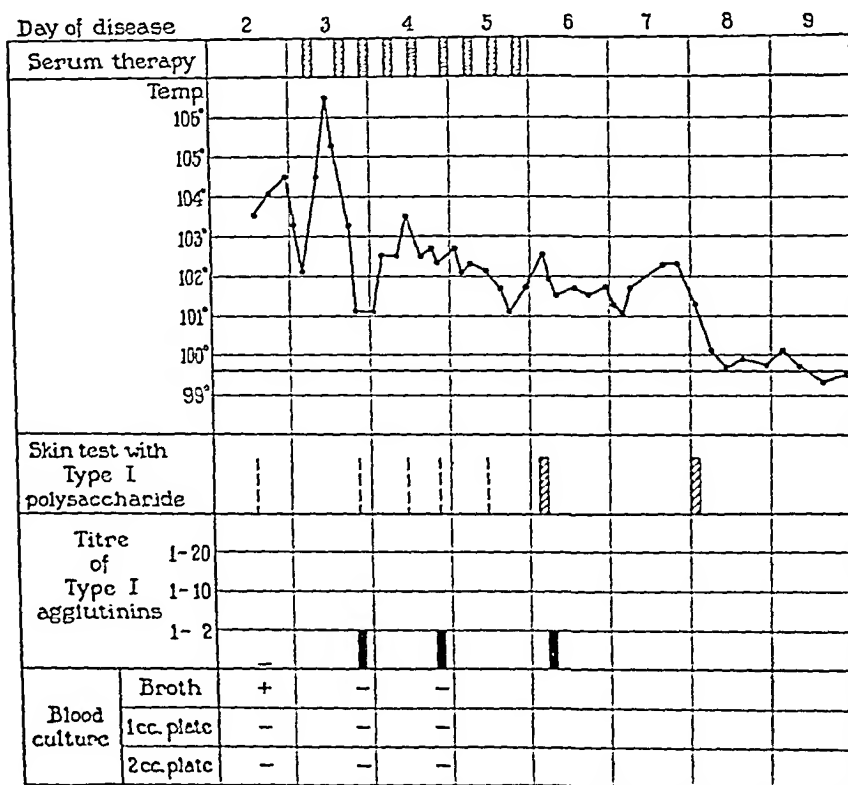


CHART 3. Patient H. (Hospital No. 7165). Type I pneumococcus pneumonia and septicemia.

serum treatments, the pulmonary lesion was spreading and the skin test remained negative. When the skin test became positive, no increase in antibody titre occurred, nor had any definite objective change occurred in the patient's condition. Experience suggested that recovery would follow the positive reaction, and that was the case. The occurrence of a positive test is obviously in this case not due

As the experiments show, of the animals sensitized with suberanic acid azoprotein, three out of five injected with 0.33 mg. of the suberanic dye died in typical anaphylactic shock, one had slight symptoms and one was negative. Distinct anaphylactic symptoms were also observed in animals which received 0.16 and 0.08 mg. of the dye. Injections of 1.3 mg. produced severe sickness and drop in tempera-

TABLE I

*Animals Tested by Intravenous Injection of Solutions of Resorcinoldisazo-*p*-Suberanic Acid*

Animals sensitized with azoprotein made from <i>p</i> -amino-suberanic acid				Animals sensitized with azoprotein made from <i>p</i> -amino-succinanic acid				Normal animals			
Guinea pig No.	Amount of dye injected	Subsequent change in body temperature	Result, symptoms	Guinea pig No.	Amount of dye injected	Subsequent change in body temperature	Result, symptoms	Guinea pig No.	Amount of dye injected	Subsequent change in body temperature	Result, symptoms
	mg.	°C.			mg.	°C.			mg.	°C.	
28	0.33		† 5 min.	16	0.66	-0.5	Negative	48	0.66	-0.5	Negative
29	0.33		† 4 "	17	0.33	-0.3	"	49	0.66	-1.2	"
30	0.33		† 6 "	18	0.33	-0.3	"	50	0.33	-0.9	"
31	0.33	-1.3	Slight								
32	0.33	+0.4	Negative								
33	0.16	-0.7	Moderate								
34	0.08	-1.2	"								
35	0.08	-1.5	Slight								

† Death of animal.

Autopsies showed the characteristic features of anaphylactic death (heart beating, lungs distended).

ture. These latter experiments have not been recorded in the table because the quantity used had toxic effects in some normal animals. The survival of animals injected with the largest dose (1.3 mg.) may be due to the zone phenomenon described by Klopstock and Selter (4), if not to an interference of the toxicity of the substance when administered in this quantity.

More uniform results were obtained by sensitization with the succinanic acid azoprotein. In this series typical anaphylactic

sively to a high titre. The blood became sterile by the 7th day, and remained sterile till death. The temperature dropped to normal on the 8th day, and signs of resolution were heard over the left lower lobe. But the type-specific skin test remained negative throughout. The patient alternated between delirium and stupor, and finally succumbed on the 10th day. At autopsy, massive pneumonic involvement of the left lung with beginning resolution, and consolidation of the right lower lobe, were found. Chronic nephritis, interstitial purulent hepatitis, and pancreatitis were also observed.

In this instance, it is clearly seen that cutaneous reactivity may be the only indication that recovery is not occurring. Normal temperature, pulse, and respirations, negative blood cultures, high titre of antibodies, and physical signs of resolution were present, but the additional factors which play a rôle in the production of a positive skin test were absent. Whether additional serum therapy would have been of aid is problematical, but on the basis of present experience it would be considered advisable.

Patient 5, Chart 5, Case 7162

The patient, a man of 27, was admitted to the hospital on the 2nd day of illness, suffering from lobar pneumonia of the left lower lobe. On admission, blood culture yielded a Type I Pneumococcus; no type-specific agglutinins were present in the patient's serum; the skin test with Type I S.S.S. was negative. Serum treatment was begun 7 hours after admission, and continued at 6 to 8 hour intervals. After the second treatment, type-specific antibodies were present in the blood, increased in titre, and remained present throughout the illness. The blood culture became negative and remained so until the day of death, when it became positive again. The pneumonic process continued to spread in the face of circulating antibodies and sterile blood so that the entire left lung and the right lower and upper lobes became involved. Cyanosis became extreme, although the patient was in the oxygen chamber. The skin tests were repeatedly and consistently negative. Eleven doses of serum were given, but death intervened on the 6th day. No autopsy was permitted.

This case presents another instance in which the serological findings would be considered favorable. Although circulating antibodies were present in a good titre, the skin tests were all negative. The same lack of response has been consistently noted in the fatal cases of the series.

The difficultly reproducible results reported previously with a dye made from *p*-aminotartranilic acid (2, 3) are easily understood on account of the weak precipitin reactions of this substance (1). In any case, these observations find confirmation in the present experiments. With regard to the somewhat irregular results obtained with the suberanilic dye, it may be pointed out that the optimum conditions for sensitization have not been studied systematically.

In the anaphylactic experiments the use of aged solutions of the dyes was suggested by the finding that such solutions are more readily precipitable (1) and in preliminary experiments with the succinilic acid dye an increase in the shocking effect was noticeable upon aging.

The fact that anaphylactic shock can be brought about with substances other than proteins has been established by Tomcsik (6, 7), and Avery and Tillett (8) who succeeded in shocking sensitized animals by the injection of bacterial carbohydrates. The same conclusion is reached from the present experiments which demonstrate that anaphylactic shock can also be produced with synthetic substances of simple constitution.

SUMMARY

Experiments are described which show that anaphylactic shock can be induced in animals sensitized with azoproteins by injecting them with azodyes containing the same azo components as the sensitizing antigen.

The anaphylactic reactions are specific and occur with quantities of the dyes as small as fractions of milligrams.

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mained at about the normal level, the blood culture had become sterile, but the skin test elicited a response so faint that it could only be considered doubtful. The following day a similar result was obtained. After the 10th day, delirium ceased and the patient improved somewhat. Convalescence was delayed by the development of an encapsulated empyema, which was drained by operation on the 25th day.

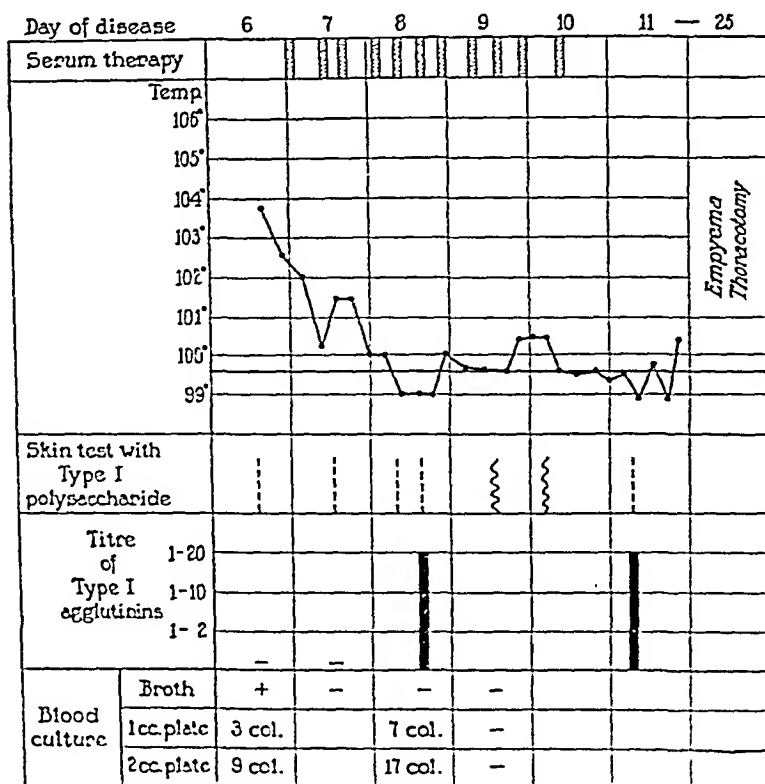


CHART 6. Patient Mu. (Hospital No. 7580). Type I pneumococcus pneumonia with septicemia and empyema.

This case is the one exception in the entire series of 53 patients in that it is the only instance in which a patient, at or about the time of recovery from the acute phase of pneumonia, failed to give a distinctly positive skin test. It is also the only case without a definitely positive reaction which did not have a fatal termination. On the 9th and 10th days, reactions were observed which were considered suggestive. It

The use of human tissue is common practice at the present time whether for diagnosis or to establish a virus strain in animals—for example the use of the spinal cord from man to convey the infection of poliomyelitis to monkeys. Much information of great value to the student of disease has come from this type of biological study of human tissue whether obtained by operation or at autopsy.

Method

The care of animals and general technique employed in the anemia colony have been described in detail elsewhere (4, 5). A good deal of work has been reported (3, 7) dealing with liver fractions containing these potent factors which promote hemoglobin formation in anemia due to bleeding in dogs. Taking advantage of this experience we have prepared the human liver tissue in such fashion as to insure the presence of these same factors in the material as finally used in the 7-day test period. The material was preserved in cold storage until used, usually for a period of between 2 and 10 weeks. We have found by many observations that the material does not deteriorate under these conditions as regards potency for new hemoglobin production. The control animal material is treated in exactly similar fashion and thus gives an accurate base line of comparison.

Iron analysis was done on samples taken from the laboratory specimens as described by Kennedy (1) and the figures expressed as milligrams per cent of Fe appear in the tables. It is obvious that the figures do not represent accurately the amount of tissue iron as the amount of contained blood is a variable factor which we cannot control in this material.

EXPERIMENTAL OBSERVATIONS

The tables below give the values for hemoglobin production as modified by the potent factors present in any given human liver. The control test on the same dog is also given in the same line and the last column gives a percentage comparison of human and animal liver. In estimating this percentage the amount of material used in each instance is introduced into the computation—for example the control intake may be 300 gm. and the human material 150 gm. If the new formed hemoglobin is the same in each instance, the percentage ratio

made within 1 to 2 hours after the administration of serum. Nevertheless, 20 of 23 Type I and Type II serum-treated cases which recovered gave positive skin reactions, while of 5 treated cases which terminated fatally, 1 gave a positive result.

In the present series of cases treated with Type I serum, a positive skin test has always been indicative of recovery, and in only 1 instance did recovery occur in the absence of a positive skin reaction. In all these cases, circulating type-specific antibodies were demonstrable at the time a positive cutaneous test was first obtained. However, reference to charts of Patients 3, 4, and 5, reveals that a positive test is not simply referable to a high concentration of circulating antibodies, since the titre of antibodies may be the same before recovery when the skin test is negative, as it is later when a positive test occurs. These charts also show that, in fatal cases, although antibodies may be present in the blood, a positive skin test is not obtained. Consequently, it appears that cutaneous reactivity to S.S.S. is not merely dependent upon the presence of circulating type-specific antibodies. On the other hand, its development is always associated with the presence of these antibodies in the blood. These observations indicate that for positive cutaneous reactivity there are required both active tissues and specific antibodies homologous in type to that of the S.S.S. injected, and that absence of either factor results in failure to react.

Recovery from pneumonia is a complicated process in which cellular activities play an important rôle. This is strikingly demonstrated in the study of resolution of pneumonia. A positive skin test to S.S.S. is also invariably associated with recovery, and it is suggested that this reaction may be an indicator of a general reawakening of cellular activity in the presence of type-specific antibodies. The failure to obtain a positive reaction in fatal cases, or in cases before recovery begins, even when type-specific antibodies are present, may be attributable to a depression of general cellular activity by the toxic factors of disease. This interpretation is supported by the observation of Finland and Sutliff (6) that when a Type I patient is treated with serum containing both Type I and Type II antibodies, the skin test to Type II S.S.S. does not become positive before the Type I S.S.S. skin test, although Type II antibodies have been present in relatively high titre throughout the course of therapy.

Liver—laboratory specimen 1380 gm.

Histological specimen—liver lobules and cells are normal. Lipochrome pigment is abundant. Fat droplets are visible within the liver cells. Kupffer cells normal.

X-670. Normal male—cerebral trauma—skull fracture and hemorrhage—45 yrs.

Liver—laboratory specimen 1800 gm.

Histological specimen—granular, frothy liver cells (glycogen). Slight lipochrome, slight congestion, few mononuclears in portal tissue. Practically normal.

TABLE 1

*Hemoglobin Production Factors in Normal Human Liver
Sudden Death from External Trauma*

Number	Cause of death	Iron content human liver		Liver intake per day		Hemoglobin output per 7 days feeding		
		fresh tissue	daily intake	Human	Control	from Human	from Control	ratio Human to Control
		mg. per cent	mg.	gm.	gm.	gm.	gm.	per cent
X-3011	Automobile	12.8	31	240	300	57	35	204
X-2410	Automobile	25.0	43	173	300	39	44	177
X-2722	Trauma	14.1	31	210	300	54	45	169
X-2724	Automobile	7.7	16	200	300	47	54	131
X-2720	Trauma	8.2	24	290	300	41	46	91
X-2579	Automobile	9.1	30	330	200	89	62	87
X-2580	Trauma	9.3	18	195	300	80	52	235
X-670	Trauma	—	—	257	300	33	26	150
X-357	Trauma	—	—	286	300	69	34	216
Average		12.3	28	242				162

X-357. Large strong male—fracture of skull and hemorrhage (accident)—50 yrs.

Liver—laboratory specimen 2000 gm.

Histological specimen—liver cells are large (glycogen). No necrosis. Lipochrome abundant. Acute congestion.

Table 1 shows the results of biological analysis of 9 normal cases, death supervening in a few hours after lethal external trauma. There is considerable individual variation in the content of these human livers but it is obvious that the concentration of the factors influencing hemoglobin regeneration is definitely greater in the human liver as compared with the animal control—162 for the human and 100 for the control. This difference may be due to food factors.

The iron content of fresh liver tissue is 12.3 mg. per cent and only

examples of the occurrence of agglutinins in cases terminating fatally and in which the skin test, by remaining consistently negative, prognosticated the fatal outcome. In Chart 6, a good titre of agglutinins was present although the septicemia was increasing. The skin test became only doubtfully positive at time of recovery, but this may have been due in part to the fact that the skin of the negro made the result more difficult to read. When a positive cutaneous reaction has been obtained, however, recovery has always ensued. As additional advantages, the skin test has simplicity of technic, the results are easily read, it is rarely equivocal, the answer is obtained in 15 to 30 minutes, the results have been remarkably regular, and it apparently measures the resultant of antibody and tissue activity. The results of this study appear to establish the value of the skin test with Type I S.S.S. in the serum treatment of Type I pneumococcus pneumonia.

SUMMARY AND CONCLUSIONS

Skin tests were made with Type I S.S.S. in 53 cases of Type I pneumococcus lobar pneumonia, 48 of which were treated with antipneumococcus Type I serum. In all but 1 of the 46 recovered cases a positive, immediate skin reaction was obtained at about the time of recovery. In 7 fatal cases reactions were consistently negative, even in the presence of circulating type-specific antibodies.

The skin test has proved to be an extremely valuable guide to serum therapy, and a definite prognostic aid. The test has distinct advantages over the agglutination reaction in that it is not merely an index of circulating antibodies. When positive, it invariably denotes that recovery has begun; when negative, it indicates further serum therapy. The mechanism of the positive skin test is closely related to that operative in recovery from pneumonia, and is apparently the resultant of antibody and tissue activity.

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A-1466. Myasthenia gravis—normal liver—hemoglobin 100 per cent—56 yrs.
Liver—autopsy weight 1680 gm.—laboratory specimen 1330 gm.
Histological specimen—liver cells are normal. Lipochrome pigment in moderate amount is present in some liver cells. Kupffer cells normal.

A-390. Arteriosclerosis—senility—88 yrs.
Liver—laboratory specimen 1360 gm.
Histological specimen—liver cells show some atrophy, scattered fat droplets and moderate lipochrome in portal region. Slight congestion present.

TABLE 2

Hemoglobin Production Factors in Human Liver Tissue
Viscera Normal? Arteriosclerosis, Senility

Number	Cause of death	Iron content human liver		Liver intake per day		Hemoglobin output per 7 days feeding		
		fresh tissue	daily intake	Human	Control	from Human	from Control	ratio Human to Control
		mg. per cent	mg.	gm.	gm.	gm.	gm.	per cent
A-1096	Aneurism	10.5	23	220	200	53	62	78
A-1257	Cardiac	7.3	15	200	300	33	53	94
X-2642	Brain hemorrhage	7.3	19	260	300	36	38	109
X-2578	Cyanide	13.9	32	230	300	71	53	173
X-2542	Heat stroke	—	—	380	300	71	67	84
X-2506	Trauma	16.1	22	130	300	13	30	100
A-1203	Pneumothorax	6.3	12	190	300	19	30	100
A-1466	Myasthenia	20.2	38	189	300	60	67	143
A-390	Senility	—	—	194	300	35	33	167
A-81	Arteriosclerosis	—	—	224	225	31	36	86
A-1185	Arteriosclerosis	—	—	185	300	36	38	157
Average.		11.7	21	218				117

A-81. Arteriosclerosis—encephalomalacia—terminal bronchopneumonia—76 yrs.
Liver—laboratory specimen 1570 gm.
Histological specimen—atrophy of liver cells is conspicuous and a few fat droplets are seen. Slight cell infiltration of portal tissue. Central liver cells very small. Lipochrome abundant.

A-1185. Arteriosclerosis—coronary occlusion—hemoglobin 80 per cent—73 yrs.
Liver—laboratory specimen 1300 gm.
Histological specimen—liver shows a senile type of atrophy with increase of lipochrome pigment in the hepatic epithelium. A few fat droplets are present in liver

ANAPHYLACTIC SHOCK BY AZODYES

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In view of the parallelism existing between the phenomena of specific precipitation and anaphylaxis, the observation that certain azodyes give typical precipitin reactions (1) led us to investigate the possibility of inducing anaphylactic shock by such dyes in animals sensitized to azoproteins. In fact, suggestive results along this line had been obtained (2, 3), with animals sensitized to azoproteins, prepared from *p*-aminotartranilic acid, but these effects were not constant and could not be reproduced easily.

For the experiments to be described, azodyes were used which were obtained by coupling resorcinol with diazotized *p*-aminosuccinanilic acid and *p*-aminosuberanilic acid.

EXPERIMENTAL

The antigens used for sensitization were prepared from horse globulin and the diazotized amino acids in the manner already described (1). Guinea pigs weighing 250 to 300 gm. received an intraperitoneal injection of 1 cc. of a 1 per cent solution of the azoprotein and 1 week later a second injection of the same quantity. The animals were tested 3 weeks after the last injection.

For the anaphylactic experiments resorcinoldisazo-*p*-succinanilic acid and resorcinoldisazo-*p*-suberanilic acid were used; both dyes had been analyzed and gave values for nitrogen corresponding to their chemical formulas (1). Stock solutions were prepared by dissolving 1/25 millimol of the dye (corresponding to 21.9 mg. of resorcinoldisazo-succinanilic acid and 26.4 mg. of resorcinoldisazo-suberanilic acid) in 6 cc. *N*/50 sodium hydroxide and adding 2 cc. *N*/50 hydrochloric acid and 2 cc. distilled water. After centrifuging to remove traces of undissolved material, the dye solutions were kept in the ice box for 1 week in tubes of pyrex glass, closed with rubber stoppers. The tests were made by intravenous injection of 1 cc. of various dilutions of the stock solutions, which were made isotonic by addition of the required amount of salt solution.

The results are presented in Tables I and II.

Histological specimen—liver shows cloudy swelling and very little fat. Lipochrome pigment moderate in amount in liver cells. Kupffer cells enlarged but show no pigment.

A-1467. Scarlet fever—bronchopneumonia—hemoglobin 105 per cent—33 yrs. Liver—autopsy weight 2200 gm.—laboratory specimen 1780 gm. Histological specimen—liver epithelium is normal except for cloudy swelling and moderate amount of lipochrome pigment. Kupffer cells normal.

TABLE 3
*Hemoglobin Production Factors in Abnormal Human Liver
Acute Infections*

Number	Cause of death	Iron content human liver		Liver intake per day		Hemoglobin output per 7 days feeding		
		fresh tissue	daily intake	Human	Control	from Human	from Control	ratio Human to Control
		mg. per cent	mg.	gm.	gm.	gm.	gm.	per cent
A-187	Erysipelas	—	—	224	300	17	28	81
A-223	Erysipelas	—	—	320	400	30	39	97
A-222	Erysipelas	—	—	330	400	22	39	69
A-186	Lung abscess	—	—	188	300	47	34	224
A-1465	Scarlet	17.0	46	270	200	96	62	114
A-1467	Scarlet	8.3	21	250	300	65	35	224
A-195	Endometritis	—	—	278	300	31	40	84
A-174	Peritonitis	—	—	161	300	12	41	55
A-221	Pneumonia	—	—	421	225	37	33	60
X-352	Influenza	—	—	300	225	47	33	107
A-112	Typhoid	—	—	243	300	41	30	171
Average				271				117

A-195. Endometritis—streptococcus—septicemia—icterus—20 yrs. Liver—laboratory specimen 1950 gm. Histological specimen—liver cells show extreme acute fatty degeneration. There are scattered necrotic liver cells and a few nests of mononuclears. Slight lipochrome. Moderate congestion. No definite icterus. This picture resembles phosphorus poisoning.

A-174. Bladder perforation—general peritonitis 5-6 days duration—50± yrs. Liver—laboratory specimen 1130 gm. Histological specimen—liver cells are nearly normal but for slight cloudy swelling. There is moderate capsular fibrosis and portal increase in stroma. Lipochrome slightly increased.

death occurred in all animals reinjected with 1.1 and 0.55 mg. of the succinanilic dye and in three of the five guinea pigs injected with 0.27 and 0.07 mg.; even 0.035 mg. of the dye still produced marked anaphy-

TABLE II

*Animals Tested by Intravenous Injection of Solutions of Resorcinoldisazo-*p*-Succinanilic Acid*

Animals sensitized with azoprotein made from <i>p</i> -amino-succinanilic acid				Animals sensitized with azoprotein made from <i>p</i> -amino-suberanilic acid				Normal animals			
Guinea pig No.	Amount of dye injected	Subsequent change in body temperature	Result, symptoms	Guinea pig No.	Amount of dye injected	Subsequent change in body temperature	Result, symptoms	Guinea pig No.	Amount of dye injected	Subsequent change in body temperature	Result, symptoms
	mg.	°C.			mg.	°C.			mg.	°C.	
1	1.1		† 4 min.	21	2.2	-0.3	Negative	41	2.2	-1.1	Negative
2	1.1		† 5 "	22	2.2	-0.4	"	42	2.2	-0.1	"
3	1.1		† 5 "	23	1.1	+1	"	43	2.2	-1.3	"
4	1.1		† 21 "	24	1.1	-0.5	"	44	1.1	-1	"
5	1.1		† 4 "	25	1.1	-0.8	Slight	45	1.1	-0.7	"
6	1.1		† 4 "	26	0.55	-0.1	Negative	46	1.1	-0.8	"
7	0.55		† 5 "	27	0.27	-1.3	"	47	1.1	-0.1	"
8	0.55		† 4 "								
9	0.27		† 4 "								
10	0.27		† 4 "								
11	0.27	-0.6	Slight								
12	0.07		† 5 min.								
13	0.07	-2.9	Severe								
14	0.035	-1.8	"								
15	0.035	-0.7	Slight								

† Death of animal.

Autopsies showed the characteristic features of anaphylactic death (heart beating, lungs distended).

lactic symptoms. Consequently the quantities necessary for eliciting shock are of the same order of magnitude as those required when proteins are used (5), in conformity with the sensitivity of the precipitin reaction of the dyes (1). The doses used and still larger ones had practically no effect upon normal guinea pigs or animals sensitized with a heterologous azoprotein.

droplets in moderate amount in liver cells. Central lipochrome in moderate amount, no necroses.

X-509. Cystitis and pyelonephritis—uremia—49 years.

Liver—laboratory specimen 1620 gm.

Histological specimen—granular swollen liver cells. Leucocytes and polymorphonuclears in some portal areas. Little lipochrome.

A-1794. Diabetes—coma—hemoglobin 90 per cent—44 yrs.

Liver—autopsy weight 2270 gm.—laboratory specimen 1900 gm.

Histological specimen—liver sections practically normal; the liver cells show a frothy protoplasm suggesting abundant glycogen. Lipochrome pigment scanty.

A-1768. Diabetes—septicemia—coma—51 yrs.

Liver—autopsy weight 1900 gm.—laboratory specimen 1700 gm.

Histological specimen—liver sections essentially normal but for slight parenchymatous change.

A-1327. Diabetes—portal thrombosis—hemoglobin 40 per cent—73 yrs.

Liver—autopsy weight 1450 gm.—laboratory specimen 1200 gm.

Histological specimen—liver cells are swollen and show some fat droplets. No pigment is seen. The portal stroma is increased and there is a little annular cirrhosis. A few small hyaline necroses are found. Kupffer cells normal.

A-1940. Thyrotoxicosis—basal metabolism +56 per cent—46 yrs.

Liver—autopsy weight 1150 gm.—laboratory specimen 1100 gm.

Histological specimen—liver lobules relatively normal. Some liver cells contain a few small fat droplets. Lipochrome is pretty abundant in the center of the lobule. Kupffer cells are normal.

A-1457. Thyroid adenoma with intoxication—hemoglobin 80 per cent—50 yrs.

Liver—autopsy weight 1080 gm.—laboratory specimen 900 gm.

Histological specimen—liver cells show some atrophy and increase in lipochrome pigment—otherwise normal. Kupffer cells normal.

A-1285. Hyperthyroidism and psychosis—embolism—hemoglobin 95 per cent—59 yrs.

Liver—autopsy weight 1350 gm.—laboratory specimen 1325 gm.

Histological specimen—liver lobules practically normal. A few fat droplets seen at margin of lobule and lipochrome pigment in the central portion. Kupffer cells normal.

A-1211. Thyrotoxicosis—hemoglobin 60 per cent—64 yrs.

Liver—laboratory specimen 1110 gm.

HEMOGLOBIN PRODUCTION FACTORS IN THE HUMAN LIVER

I. NORMAL, INFECTION AND INTOXICATION

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When it was found (6) that *beef liver* contained potent factors for hemoglobin regeneration in anemia due to bleeding in dogs, it was logical that a study of various liver material should follow. It was soon found (2) that fish liver was practically inert but that the liver tissue of many warm blooded animals contained these potent factors in abundance. Liver tissues from the pig, sheep, beef, reindeer, horse and chicken have been thoroughly tested and in general there is a remarkable constancy in the reaction of a given standardized anemic dog to animal liver feeding. In general we may say that the feeding of 300 gm. pig liver per day for 2 weeks, will effect the output of approximately 100 gm. new hemoglobin above the control level in these anemic dogs. Dogs have individual capacities of hemoglobin production which must be known before accurate standardization is established.

We have often speculated as to the concentration of these potent factors for hemoglobin regeneration in the human liver, normal and abnormal, as well as in the diseased animal liver. As opportunities presented during the past 7 years¹ we have tested out these potent factors using our colony of carefully standardized anemic dogs.

It is obvious that the behavior of these potent factors for hemoglobin regeneration holds much of interest for the student of pigment metabolism as well as for the physician who would understand the therapy of anemia.

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A-68. Ulcerative colitis—ileostomy—cystitis—bronchopneumonia—hemoglobin 65 per cent—40 + yrs.

Liver—laboratory specimen 1500 gm.

Histological specimen—liver cells show periportal fatty degeneration, 3/4 of the liver cells are normal. Lipochrome abundant.

A-162. Colitis—general peritonitis—empyema—pneumonia and abscess—long continued suppuration for 2 months—63 yrs.

Liver—autopsy weight 1600 gm.—laboratory specimen 1400 gm.

Histological specimen—perihepatitis with organizing periportal fibrosis of moderate degree. Occasional small necroses. A little fat in periportal liver cells. Slight central atrophy with much lipochrome. Slight central congestion.

A-1751. Chronic arthritis—arteriosclerosis—bronchopneumonia—hemoglobin 60 per cent—67 yrs.

Liver—autopsy weight 1370 gm.—laboratory specimen 1250 gm.

Histological specimen—liver cells show atrophy and a few fat droplets. Lipochrome pigment is well marked. There are central hyaline necroses in many lobules. These areas of injury may involve 10 per cent of the liver parenchyma examined.

Table 4 shows a considerable group of chronic intoxications in which abnormalities of the kidney, pancreas and thyroid are represented. The potent hemoglobin factors in these livers are present in high concentration which approximates the high values for the normal given in Table 1. This is 149 per cent for Table 4 and 162 per cent for Table 1. In *uremia* the figures for these hemoglobin factors are uniform and all seem close to the general average of 149. In connection with the anemia usually present in chronic nephritis we may refer to Table 33 (Paper III) in which the values for extreme secondary anemia are given.

Diabetes evidently is often associated with rather low liver values for hemoglobin producing factors—in the 3 cases given an average of 99 per cent as compared with the general average of 149 per cent (Table 4). Some observers might wish to explain this on the basis of an interrelation of liver and pancreatic functions.

Thyroid abnormalities show very wide fluctuations in the content of hemoglobin producing factors in these livers. Some values are unusually low and one extraordinarily high. This case A-1457, Table 4, shows nothing very unusual upon which to base an explanation of

will be 200 per cent for the human material which obviously is twice as potent.

The control base line in these experiments is established by standard intakes of pig liver in amounts per day as indicated in the tables.

Table 1. *Diagnosis and histological description of liver.*

X-3011. Normal male—automobile accident—21 yrs.

Liver—autopsy weight 1850 gm.—laboratory specimen 1680 gm.

Histological specimen—liver lobules and epithelium normal; a little lipochrome pigment is seen.

X-2410. Normal male—automobile accident—fractured skull—44 yrs.

Liver—autopsy weight 1410 gm.—laboratory specimen 1210 gm.

Histological specimen—liver lobules normal; liver cells normal but for a very heavy deposit of lipochrome pigment. Careful study shows no material which gives iron stain. The iron content of this liver is unusually high but it is all within the cell protoplasm not in pigment granules. This is probably due to dietary factors.

X-2722. Normal male—fatal trauma—50 yrs.

Liver—autopsy weight 1690 gm.—laboratory specimen 1550 gm.

Histological specimen—liver cells and lobules normal; lipochrome is present in moderate amounts.

X-2724. Normal male—automobile accident—57 yrs.

Liver—autopsy weight 1550 gm.—laboratory specimen 1470 gm.

Histological specimen—liver cells and lobules are normal. Few liver cells show fat droplets and some lipochrome pigment.

X-2720. Normal male—trauma and death in 10 hrs.—60 yrs.

Liver—autopsy weight 2200 gm.—laboratory specimen 2050 gm.

Histological specimen—liver cells and lobules normal. A few mononuclears appear in the portal stroma. Lipochrome is abundant in the central portion of the lobules.

X-2579. Normal elderly female—obesity—automobile accident—65 yrs.

Liver—autopsy weight 2350 gm.—laboratory specimen 2320 gm.

Histological specimen—the liver cells show many large fat droplets, a condition obviously related to the obesity. No pigment found. Kupffer cells normal.

X-2580. Normal elderly male—trauma and death in 12 hrs.—hemoglobin 105 per cent—64 yrs.

swelling of the liver. For example a liver weighing 2100 gm. and testing 100 per cent (Table 3) represents 50 gm. potential hemoglobin as tested by the usual biological assay.

In the literature of liver feeding in experimental anemia there has been much written about the importance and potency of *organic iron* as it is found in the liver parenchyma. Some authors would have us believe that the potency of the liver is dependent upon this contained organic iron and that the blood regeneration following liver feeding is a true index of the content of organic iron. Plenty of evidence is submitted in these papers to show that liver potency may be high when the iron analysis is very low and *vice versa*. For example pig liver is rated as 100 per cent or the normal control level and it contains 19 mg. per cent Fe. Human liver in normal cases rates at 162 per cent potency yet the iron analysis shows but 12 mg. per cent. In secondary anemia due to bleeding (Table 33, Paper III) the liver shows a potency close to normal (135 per cent) and the iron analysis reads 5.3 mg. per cent. In other words we may observe complete dissociation of the iron content and the concentration of hemoglobin production factors within the liver.

SUMMARY

Human liver tissue has been assayed to determine the concentration of hemoglobin production factors in normal and abnormal states. Standardized dogs made anemic by bleeding have been used in this biological assay and the human liver tissue compared with control animal tissue.

Normal human liver tissue (external trauma) contains much more of these hemoglobin production factors than the normal control animal liver—the ratio being 162 to 100.

In this form of biological assay 42 gm. of animal liver or 26 gm. of human liver represent 1 gm. of potential hemoglobin.

A second group (Table 2) in which the viscera were practically normal except for atrophy, the cases presenting a good deal of arteriosclerosis and senile changes, shows a lower content of these hemoglobin production factors. The ratio of human to control here is 117 to 100. This is certainly the low limit of normal.

Acute fulminant infections reduce somewhat the store of these po-

one case deviates conspicuously from this average. The reason for this individual variation is not clear. It is at least possible that this high value may be due to diet factors especially rich in iron. The control animal liver contains on the average 19 mg. per cent Fe.

Table 2. *Diagnosis and histological description of liver.*

A-1096. Aneurism rupture—liver normal—55 yrs.

Liver—laboratory specimen 1560 gm.

Histological specimen—normal liver tissue; a little lipochrome pigment seen in hepatic epithelium.

A-1257. Sudden cardiac death—liver normal—hemoglobin 90 per cent—23 yrs.

Liver—autopsy weight 1550 gm.—laboratory specimen 1430 gm.

Histological specimen—liver is practically normal. There are a few mononuclears in the periportal tissue. The liver cells show some lipochrome pigment. Kupffer cells are normal.

X-2642. Normal adult—cerebral hemorrhage—alcoholic?—40 yrs.

Liver—autopsy weight 1900 gm.—laboratory specimen 1840 gm.

Histological specimen—all liver cells show fat droplets, usually of large size. This suggests alcoholism. No excess pigment. No liver necrosis.

X-2578. Normal male—suicide—cyanide—50 yrs.

Liver—autopsy weight 1800 gm.—laboratory specimen 1640 gm.

Histological specimen—liver cells normal. A moderate amount of lipochrome pigment found in liver cells of the central part of the lobules. Kupffer cells normal.

X-2542. Heat stroke—obesity—50 yrs.

Liver—laboratory specimen 2670 gm.

Histological specimen—there is some fatty degeneration but liver cells in general are normal. There is a moderate increase in portal connective tissue. Eosinophiles are numerous in liver capillaries. No pigment of any type seen. Kupffer cells normal.

X-2506. Trauma—fat embolism—hemoglobin 90 per cent—80 yrs.

Liver—autopsy weight 960 gm.—laboratory specimen 940 gm.

Histological specimen—liver sections typical of senile atrophy. Lipochrome pigment is abundant in liver cells particularly in the center of the lobules. Kupffer cells normal.

A-1203. Acute pneumothorax—normal viscera—40 \pm yrs.

Liver—autopsy weight 1390 gm.—laboratory specimen 1340 gm.

Histological specimen—liver quite normal except for capillary congestion. No pigment to be seen.

cells. Kupffer cells normal. A few liver cells here and there contain a few pigment granules giving an iron stain.

Table 2 shows a group of human cases in which the viscera are relatively normal but show a variety of concomitant abnormalities. The liver itself is practically normal except for senile atrophy in many cases. Death took place suddenly in some cases or after a few hours in others. Arteriosclerosis is present in the majority of these cases.

The hemoglobin production factors in this liver tissue are obviously less abundant than in the normal liver material of Table 1. Even in these cases (Table 2) the potent factors are more abundant in the human liver than in animal liver—117 to 100. This figure of 117 may appear as the low normal or if we insist that Table 1 is the actual normal then these chronic senile changes may be in part responsible for the drop in the liver content of potent hemoglobin production factors.

Table 3. *Diagnosis and histological description of liver.*

A-187. Acute erysipelas and toxic death—66 yrs.

Liver—autopsy weight 1850 gm.—laboratory specimen 1570 gm.

Histological specimen—swollen liver cells, much lipochrome in center of lobules, clusters of mononuclears in portal areas. No fat. Slight congestion.

A-223. Erysipelas—endocarditis—acute nephritis—60 yrs.

Liver—laboratory specimen 2250 gm.

Histological specimen—swollen liver cells, few small necroses and nests of leucocytes, few fat droplets, slight inflammation of bile ducts and considerable portal inflammation and fibrosis. Little lipochrome pigment.

A-222. Erysipelas—secondary subacute nephritis—48 yrs.

Liver—laboratory specimen 2300 gm.

Histological specimen—liver cells swollen, few fatty cells, few tiny necroses, mononuclears and polymorphonuclears in portal tissue. Little lipochrome, moderate congestion, numerous polymorphonuclears in capillaries.

A-186. Erysipelas 3 wks. ago—lung abscess—arteriosclerosis—pachymeningitis—68 yrs.

Liver—autopsy weight 1570 gm.—laboratory specimen 1320 gm.

Histological specimen—liver cells are granular and swollen—a few fat droplets seen. Lipochrome present—no necrosis, slight congestion. A good many mononuclears found in portal areas.

A-1465. Scarlet fever—bronchopneumonia—hemoglobin 95 per cent—49 yrs.

Liver—autopsy weight 2000 gm.—laboratory specimen 1900 gm.

A-1226. Heart disease—passive congestion of liver—hemoglobin 70 per cent—44 yrs.

Liver—autopsy weight 2200 gm.—laboratory specimen 1840 gm.
Histological specimen—liver lobules show central atrophy as usual. These cells show some fat droplets and lipochrome pigment. The liver cells in the periportal regions are normal. Kupffer cells not pigmented. A few bile canaliculi in center of lobules show brown colloid.

A-1249. Heart disease—passive congestion of liver—hemoglobin 93 per cent—53 yrs.

Liver—autopsy weight 1900 gm.—laboratory specimen 1100 gm.
Histological specimen—extreme passive congestion with complete central atrophy of liver cells here. In center of lobule phagocytes full of lipochrome pigment are numerous but mainly an empty reticulum is observed. In mid zone a few liver cells show fat droplets. The liver cells in the outer half of each lobule appear normal.

A-94. Old endocarditis of all valves—passive congestion of liver—41 yrs.

Liver—laboratory specimen 1240 gm.
Histological specimen—liver lobules show extreme passive congestion with central atrophy and some cell necrosis. Bile canaliculi conspicuous in mid zone of lobule. Portal tissue normal. About $\frac{1}{3}$ liver parenchyma looks normal. Lipochrome present.

A-78. Rheumatic heart with decompensation—passive congestion of liver—41 yrs.

Liver—laboratory specimen 1370 gm.
Histological specimen—liver lobules show early acute passive congestion with atrophy and central necrosis, about $\frac{2}{3}$ liver cells look normal. Little fat, no lipochrome. In some sections the liver lobules show necrosis involving almost $\frac{1}{2}$ the liver cells.

A-60. Syphilitic aortitis—cardiac insufficiency—passive congestion—broncho-pneumonia—45 yrs.

Liver—laboratory specimen 1340 gm.
Histological specimen—liver cells very small. Central atrophy present, a good deal of fat, no necroses. Lipochrome abundant. Few leucocytes.

Table 21 shows typical instances of cardiac *passive congestion* with "red atrophy" of the liver. Biological assay of this liver tissue shows subnormal or low normal values and this may be explained by simple atrophy and disappearance of many liver cells.

Table 21 shows one very unusual case—the last one in the table—A-60. There was nothing unusual about this case clinically—a man

A-221. Lobar pneumonia, Type I—39 yrs.

Liver—laboratory specimen 2950 gm.

Histological specimen—liver cells swollen and granular, few tiny fat droplets, few tiny focal necroses. Lipochrome moderate, slight portal fibrosis with mononuclears included.

X-352. Acute hemorrhagic bronchopneumonia (influenza)—30+ yrs.

Liver—laboratory specimen 2150 gm.

Histological specimen—diffuse fatty degeneration of liver cells, stroma normal. Lipochrome abundant.

A-112. Typhoid, 24 days duration—terminal bronchopneumonia—17 yrs.

Liver—laboratory specimen 1700 gm.

Histological specimen—typical numerous focal necroses, mononuclears abundant in portal tissue, liver cells swollen and granular.

Table 3 shows a group of *acute infections* in which the fever and intoxication lasted from 1 to 4 weeks. It is obvious that the potent factors for hemoglobin production are not present in high concentration in these livers. The average figure 117 per cent is identical with the average of Table 2—to be designated as subnormal or at least a low normal. In the more acute fulminating infections the values appear to be lower than in the infections which are prolonged over a few weeks. This is in harmony with Table 4.

Table 4. *Diagnosis and histological description of liver.*

A-1682. Nephritis—diabetes—uremia—52 yrs.

Liver—autopsy weight 1430 gm.—laboratory specimen 1250 gm.

Histological specimen—liver in general normal. There is a little lipochrome pigment in some of the liver cells. Kupffer cells show an occasional tiny yellow grain of pigment.

X-354. Mercury poisoning—necrosis of renal epithelium—colitis—uremia—25 ± yrs.

Liver—laboratory specimen 1570 gm.

Histological specimen—liver cells show cloudy swelling; no necroses but many mitoses seen. No leucocytes. Slight congestion. Lipochrome scanty.

A-204. Tuberculosis of kidney—pyelonephritis—emaciation—hemoglobin 65 per cent—56 yrs.

Liver—autopsy weight 900 gm.—laboratory specimen 740 gm.

Histological specimen—liver epithelium is relatively normal. Periportal large fat

and phagocytes. We may say that the normal liver parenchyma is "diluted" by this much inert tissue. We believe the remaining liver cells contain a normal amount of the substances which promote hemoglobin formation under experimental conditions.

Table 22. *Diagnosis and histological description of liver.*

A-580. Chronic pulmonary tuberculosis—amyloidosis—hemoglobin normal—23 yrs.

Liver—laboratory specimen 2700 gm.

Histological specimen—extreme amyloidosis, $2/3$ – $3/4$ of each liver lobule is amyloid. Liver cells show atrophy and fat droplets.

A-326. Chronic pulmonary tuberculosis—amyloidosis—41 yrs.

Liver—laboratory specimen 2380 gm.

Histological specimen—extreme amyloidosis, only about $1/4$ of section is hepatic epithelium. Small atrophic threads of liver cells. Few tubercles. Lipochrome conspicuous in atrophic liver cells. Very little fat.

A-348. Chronic pulmonary tuberculosis and amyloidosis—43 yrs.

Liver—laboratory specimen 2550 gm.

Histological specimen—amyloid makes up about $1/2$ of each lobule. Liver cells outside of this amyloid deposit are well preserved. No fat, no necrosis, little lipochrome.

A-226. Chronic pulmonary tuberculosis—amyloidosis—disseminated tubercles—20 yrs.

Liver—autopsy weight 1250 gm.—laboratory specimen 1220 gm.

Histological specimen—amyloid and stroma make up about $1/2$ of each liver lobule, most of the amyloid appears in portal zone, small amount of lipochrome in central half of lobule. Liver cells atrophic, little fat.

A-293. Chronic pulmonary tuberculosis—slight amyloidosis—50–60 per cent hemoglobin—22 yrs.

Liver—laboratory specimen 950 gm.

Histological specimen—fat droplets conspicuous in peripheral half of lobule. Center liver cells are swollen. Little lipochrome. Few lobules show early amyloid deposit. Few tubercles, slight portal fibrosis. Mononuclears numerous in capillaries. Kupffer cells large.

A-1359. Alcoholism—pulmonary tuberculosis—hemoglobin 90 per cent—41 yrs.

Liver—autopsy weight 2580 gm.—laboratory specimen 2350 gm.

Histological specimen—practically all liver cells show large and small fat droplets.

Histological specimen—liver cells in centers of lobules show moderate amounts of lipochrome pigment. Liver cells in peripheral half of lobules show much fatty degeneration. Kupffer cells normal.

A-64. Hyperthyroidism—organizing pericarditis—cardiac hypertrophy—56 yrs. Liver—laboratory specimen 1260 gm.

Histological specimen—liver cells show atrophy, slight lipochrome pigmentation; there is slight portal fibrosis.

TABLE 4

Hemoglobin Production Factors in Abnormal Human Liver
Uremia, Diabetes, Thyroid States, Colitis, Arthritis

Number	Cause of death	Iron content human liver		Liver intake per day		Hemoglobin output per 7 days feeding		
		fresh tissue	daily intake	Human	Control	from Human	from Control	ratio Human to Control
		mg. per cent	mg.	gm.	gm.	gm.	gm.	per cent
A-1682	Uremia	9.3	17	178	300	43	49	148
X-354	Uremia	—	—	224	300	32	35	123
A-204	Pylonephritis	—	—	105	400	18	39	180
X-509	Uremia	—	—	230	400	37	39	168
A-1794	Diabetes	11.6	31	270	300	87	72	134
A-1768	Diabetes	8.6	21	240	300	42	58	91
A-1327	Diabetes	4.3	7	170	300	19	46	73
A-1940	Toxic thyroid	9.1	14	160	300	40	58	129
A-1457	Toxic thyroid	13.7	18	125	300	62	35	413
A-1285	Toxic thyroid	9.4	18	185	300	34	67	83
A-1211	Toxic thyroid	14.5	23	158	142	47	38	112
A-64	Toxic thyroid	—	—	180	100	26	20	72
A-373	Myxedema	—	—	158	200	67	62	137
A-68	Colitis	—	—	214	300	23	33	96
A-162	Colitis	—	—	200	300	61	34	265
A-1751	Arthritis	23.8	42	178	300	86	87	165
Average.		11.6	21	186				149

A-373. Myxedema—fibrous thyroid—basal metabolism = minus 43 per cent—62 yrs.

Liver—autopsy weight 1475 gm.—laboratory specimen 1100 gm.

Histological specimen—liver cells show a good deal of atrophy with central lipochrome pigmentation. There are scattered fat droplets and a good deal of yellow pigment in Kupffer cells. Much arteriosclerosis of small arteries. Iron stain positive in pigment in Kupffer cells, not in liver cells.

Histological specimen—fatty liver cells contain large and small droplets. Moderate lipochrome, no fibrosis.

Table 22 shows typical cases of *amyloidosis* due to long continued tuberculosis. The cases are arranged in order of the extent of the amyloid deposit. The first two cases in histological sections show about two-thirds of the section made up of amyloid. If we allow for this "dilution" of the liver cells by this amyloid deposit we arrive at figures about 3 times those given in Table 22 or high normal values. If however on the basis of normal liver weights in tuberculosis we say that there is an excess weight of amyloid amounting to 1000 gm. in each instance we would find our calculations for the remaining liver cells at about the normal level. There is no evidence that amyloid contains any factors which favor hemoglobin regeneration although most of the amyloid probably is undigested and unabsorbed.

Table 22 also shows that acute and chronic *alcoholism* does not seriously deplete the store of hemoglobin producing factors in the liver. In fact if we allow for the abnormal liver weight due to fatty degeneration we arrive at values which are close to normal.

Table 23. *Diagnosis and histological description of liver.*

A-512. Primary liver cell cancer—hemoglobin 90 per cent—no icterus—54 yrs. Liver—autopsy weight 6000 + gm.—laboratory specimen 5135 gm. Histological specimen—pigmented tumor cells numerous, much necrosis. Liver cells are very rich in pigment which contains *iron*. Tumor cells show *iron* staining pigment.

A-54-31. Liver carcinoma with cirrhosis.

Liver—autopsy weight 1760 gm.—laboratory specimen 1650 gm. Histological specimen—liver cancer originating probably from hepatic epithelium. Elsewhere the liver shows a moderate grade of annular cirrhosis. The liver cells are well preserved and appear relatively normal. Lipochrome pigment is present.

X-3788. Cancer of stomach—liver metastases—hemoglobin 100 per cent—60 yrs. Liver—autopsy weight 4690 gm.—laboratory specimen 4290 gm. Histological specimen—extensive replacement of liver cells by adeno-carcinoma. There appear to be more tumor than liver cells. Liver cells show a little atrophy especially in centers of lobules. No unusual pigment. Small amount of lipochrome pigment. Kupffer cells normal.

A-1741. Embryoma testis—liver metastases—hemoglobin 60 per cent—38 yrs. Liver—autopsy weight 1700 gm.—laboratory specimen 1460 gm.

the very high figure for liver potency—413 per cent. The case was one of very severe thyroid intoxication admitted in coma, death supervening in a few hours. There was found a slight terminal bronchopneumonia. The thyroid showed the usual histological picture. Liver normal. This may indicate some important influence of a thyroid hormone upon liver function but a satisfactory explanation is not apparent.

The last 3 cases in Table 4 show that in long continued infection and intoxication there may be no decrease in the liver content of these potent hemoglobin producing factors.

DISCUSSION

We may look at liver material as representing so many grams of potential hemoglobin which will be produced in these standard anemic dogs. When 300 gm. pig liver is fed daily for 14 days we expect on the average a return of 100 gm. hemoglobin or a ratio of 4200 to 100. That is 42 gm. of pig liver is equivalent to 1 gm. hemoglobin in this type of biological assay. When we use the ratio in Table 1 indicating that human liver is more potent than pig liver (162 to 100) we arrive at a figure of 26 gm. human liver as equivalent to 1 gm. hemoglobin as tested by biological assay.

In all these tables the potential hemoglobin values for the liver tissue are given as per gram fresh weight. Some may object that it would be better to calculate for each whole liver the total potential hemoglobin in grams which its weight represents. This figure can be readily computed from the tabulated data. It is obvious that the senile atrophic liver which may be $1/2$ – $2/3$ normal weight would show subnormal values even more apparent than the tabulated values (Table 2) if we allowed for this weight shrinkage. We may say that a normal adult human liver of 1700 gm. represents potential hemoglobin amounting to 65 gm. as tested by standard biological assay. In like manner a senile liver tested at 100 (Table 2) compared to 100 per cent control and weighing 1000 gm. represents but 24 gm. potential hemoglobin.

In like manner the figures for acute infections as given per gram of liver tissue may not mean a great loss for the whole liver but in some cases a "dilution" of the potential hemoglobin values due to the cloudy

A-359. Cancer of gall bladder—liver metastases—icterus—terminal endocarditis—78 yrs.

Liver—autopsy weight 1850 gm.—laboratory specimen 1300 gm.

Histological specimen—much tumor tissue. Much liver cell necrosis in places and post mortem change. Icterus and plugs in bile canaliculi in some areas.

Atrophy of liver cells elsewhere. Lipochrome abundant.

Table 23 shows various types of primary and secondary *cancer replacement* and invasion of the liver. There is no evidence that cancer cells even when originating from hepatic epithelium contain substances which promote hemoglobin regeneration. It appears that the largest liver specimens containing the largest mass of tumor cells give lower values per gram specimen weight. We may say these masses of cancer "dilute" the potency of the remaining liver cells just as is found with amyloid deposit (Table 22). There is no reason to suppose that the tumor material is not completely disintegrated and assimilated.

The first case (A-512) in Table 23 illustrates this "dilution" by tumor tissue. This liver weighed more than 3 times normal and we may safely assume that the liver cells made up less than the normal liver weight. If we take this fact into consideration the concentration of hemoglobin production factors instead of rating 51 per cent of control would read more than 150 per cent or normal.

The second case (A-54-31) in Table 23 shows a second instance of primary liver carcinoma where there was little increase in liver weight, yet the liver cell cancer did occupy a considerable ($1/5$ – $1/7$) bulk of the liver mass. If we allow for this "dilution" with inert tumor tissue we arrive at a figure within low normal values.

The liver cells closely associated with masses of tumor cells are usually supposed to suffer from hypothetical toxic material coming from tumor growth and disintegration. The liver cells actually show some atrophy and fatty degeneration and the tumor masses may interfere with bile circulation and blood flow through liver lobules. In spite of all this, the concentration of hemoglobin producing factors within the liver cells is not significantly reduced.

In one instance (A-456) the iron analysis shows high values for the total daily intake and this iron may account for more than half the actual hemoglobin production which would mean a low value for the liver cells even when allowance is made for the "dilution" with cancer tissue.

tent hemoglobin production factors in the human liver (Table 3). The average value is 117 as compared with 100 control but the more acute cases show the lower values.

Chronic intoxications show values which are close to the human normal—151 per cent. The liver content of hemoglobin producing factors shows very wide fluctuations in cases of thyrotoxicosis. Diabetes may be associated with rather low values.

There may be complete dissociation of the organic iron content and the concentration of hemoglobin production factors in the liver.

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stroyed with much bile duct proliferation. Fibrous tissue rich in mononuclears and polymorphonuclears. Perhaps half of section shows well preserved liver cells, little fat, much granular yellow pigment in liver cells. Iron granules stain abundantly in liver cells. Few colloid plugs in canaliculi. No hepatic insufficiency.

A-1530. Hepatitis and icterus—cardiac failure—alcoholism—hemoglobin 80 per cent—54 yrs.

Liver—autopsy weight 950 gm.—laboratory specimen 840 gm.

Histological specimen—liver does not show a characteristic cirrhosis. There is no striking distortion of liver architecture. Many liver lobules are normal except for icterus. Many lobules show extensive necroses which are healing with accumulation of mononuclears and scar tissue. There are some new formed bile ducts here. Bile canaliculi are conspicuous. Kupffer cells contain much granular pigment. There is yellow pigment in many liver cells. No pigment gives a stain for iron. This liver is not insufficient but has been subjected to focal injury in many lobules.

A-1238. Subacute hepatitis—icterus—hemoglobin 90 per cent—38 yrs.

Liver—autopsy weight 1340 gm.—laboratory specimen 1100 gm.

Histological specimen—portal tissue increased and infiltrated with mononuclears. Liver lobules not extremely distorted but show scattered necroses and diffuse cell infiltration and edema of stroma. Bile canaliculi in places conspicuous, filled with yellow colloid. Kupffer cells rich in pigment. Many liver cells, possibly 1/2 of each lobule, appear normal. This process may have begun as a cholangitis. Fibrinogen not reduced.

A-700. Liver cirrhosis—nephritis chronic—cardiac congestion—hemoglobin 75 per cent—79 yrs.

Liver—autopsy weight 2460 gm.—laboratory specimen 2180 gm.

Histological specimen—annular portal fibrosis with many mononuclears included. Little bile duct proliferation. Liver cells not disarranged but show granular degeneration and little fat. Some lobules show moderate lipochrome and others not. Kupffer cells normal.

X-835. Liver cirrhosis with hepatitis—icterus—esophageal hemorrhage—62 yrs.

Liver—autopsy weight 2670 gm.—laboratory specimen 2400 gm.

Histological specimen—advanced cirrhosis with great injury of hepatic epithelium, extreme fat, good deal of necrosis, diffuse fibrosis, much abortive bile duct proliferation—bile canaliculi all distended with colloid plugs—not much lipochrome. Severe parenchyma injury.

A-564. Liver cirrhosis (hob-nail)—syphilis—subacute nephritis—hemoglobin 80 per cent—56 yrs.

HEMOGLOBIN PRODUCTION FACTORS IN THE HUMAN LIVER

II. LIVER DEGENERATION, CANCER, CIRRHOSIS AND HEPATIC INSUFFICIENCY

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When we first took up the study of the hemoglobin production factors in abnormal livers, we were surprised to find that the great majority of these cases showed normal or perhaps low normal human values. It is obvious from a glance at the tables below that only under conditions of grave injury does the liver cell give up the unknown substances which can be used to produce new hemoglobin and red cells. Even in fatal *liver insufficiency* (Table 25) the liver cells still contain some of the factors which go to form new hemoglobin although the amount of these hemoglobin production factors may be *reduced to 1/3 normal*. By way of contrast we observe in the next paper (Tables 31 and 32) that primary and aplastic anemias show a considerable *excess storage* of these same factors within the liver.

The same methods and experimental procedures were used in these experiments as described in the preceding paper—in fact all these experiments developed during the same period and the cases were assayed as material became available but the data are arranged according to the abnormal findings.

Table 21. *Diagnosis and histological description of liver.*

A-1105. Myocardial failure—passive congestion of liver—36 yrs.
Liver—autopsy weight 2150 gm.—laboratory specimen 1850 gm.
Histological specimen—typical picture of advanced passive congestion of liver lobules. The central one-half of the lobules shows atrophy of liver cells with fatty degeneration. The marginal half of each lobule shows relatively normal liver cells. Lipochrome pigment is not abundant.

liver epithelium shows a good deal of fat and very little pigment—no icterus. Scar tissue is abundant within the lobules and mononuclears are numerous. Bile duct sprouts are present. Liver cell function would seem to be impaired.

TABLE 24

*Hemoglobin Production Factors in Abnormal Human Liver
Liver Cirrhosis—No Evidence of Liver Insufficiency*

Number	Cause of death	Iron content human liver		Liver intake per day		Hemoglobin output per 7 days feeding		
		fresh tissue	daily intake	Human	Control	from Human	from Control	ratio Human to Control
		mg. per cent	mg.	gm.	gm.	gm.	gm.	per cent
A-1589	Cirrhosis	12.3	17	140	300	51	58	190
A-1468	Cirrhosis	8.1	17	210	300	50	52	139
A-615	Cirrhosis	18.5	34	185	300	49	54	148
A-1114	Cirrhosis	3.2	8	245	300	23	45	62
A-788	Cirrhosis	16.5	30	167	142	58	38	129
A-1530	Hepatitis	4.7	6	115	300	47	46	261
A-1238	Hepatitis	5.4	8	157	300	52	54	186
A-700	Cirrhosis	3.6	11	310	300	45	43	102
X-835	Hepatitis	—	—	340	300	41	40	91
A-564	Cirrhosis	6.5	7	103	300	26	53	145
A-789	Cirrhosis	—	—	396	300	71	54	100
A-29-77	Cirrhosis	17.3	23	130	300	48	35	320
A-28-168	Cirrhosis	—	—	370	300	53	54	79
A-27-160	Cirrhosis	—	—	130	300	63	53	274
A-51-27	Cirrhosis	—	—	107	300	59	53	310
X-3334	Cirrhosis	2.6	9	320	300	65	67	90
A-197	Sl. cirrhosis	—	—	190	300	36	28	200
A-205	Sl. cirrhosis	—	—	290	300	55	53	108
A-730	Sl. cirrhosis	14.5	29	200	300	50	35	218
A-1024	Sl. cirrhosis	8.3	18	210	105	71	30	118
Average.....		9.3	17	216				164

A-197. Liver cirrhosis (hob-nail)—subacute colitis—46 yrs.

Liver—laboratory specimen 1350 gm.

Histological specimen—moderate biliary cirrhosis. Mononuclears very numerous in thick portal tissue. Moderate bile duct regeneration in places. Liver cells show scattered small fat droplets, no necroses. Few colloid plugs in bile canaliculi. Bulk of liver cells are normal.

with syphilitic aortitis and cardiac failure lasting several years. A serious break in compensation was recorded 3 weeks before death. Patient came into the hospital and died in 24 hours. The liver showed the expected passive congestion. It so happened that this material was divided and tested on two dogs. The smaller amount might not be convincing but the larger amount, 120 gm. daily, gave no evidence of any hemoglobin producing factors in this material. Only one other case in all this series shows a zero test—see Table 35 (Paper III), case of eclampsia. We have no explanation to offer but believe that methods and experimental procedure can be excluded.

TABLE 21

*Hemoglobin Production Factors in Abnormal Human Liver
Cardiac Passive Congestion—Red Atrophy of Liver*

Number	Cause of death	Iron content human liver		Liver intake per day		Hemoglobin output per 7 days feeding		
		fresh tissue	daily intake	Human	Control	from Human	from Control	ratio Human to Control
		<i>mg. per cent</i>	<i>mg.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>
A-1105	Myocarditis	18.6	50	260	300	40	53	87
A-1226	Cardiac	6.7	18	260	300	25	45	64
A-1249	Cardiac	6.9	11	155	300	44	41	210
A-94	Cardiac	—	—	177	300	25	33	132
A-78	Cardiac	—	—	181	225	19	33	70
A-60	Aortitis	—	—	70	300	0	35	0
A-60	Aortitis	—	—	120	160	0	30	0
Average.....				175				

If we exclude this last case A-60 from the average, we get an average of 112 per cent which practically coincides with the degenerations listed under Table 22. All cases listed in Table 21 show typical central atrophy of liver cells, in some cases with more or less central necrosis of liver cells. The usual mid zone of fat is found while the periportal liver cells are normal. It is not surprising that this type of liver atrophy (cardiac) should show some loss of these potent hemoglobin producing factors as a goodly percentage of liver parenchyma has vanished and its place is taken by reticulum, congested capillaries

Table 25. *Diagnosis and histological description of liver.*

A-956. Cirrhosis and hepatitis—liver insufficiency—hemoglobin 40 per cent—33 yrs.

Liver—autopsy weight 2160 gm.—laboratory specimen 2050 gm.

Histological specimen—the general picture is that of a coarse annular type of cirrhosis in which scar tissue makes up more than half of all liver sections. This scar tissue is full of mononuclear and a few polymorphonuclear cells. Only an occasional island of liver cells is normal. As a rule the islands of liver cells show focal injury and infiltration with wandering cells in large numbers. The picture throughout the liver sections is that of a diffuse subacute inflammatory reaction involving stroma and hepatic epithelium as well as bile ducts—a true diffuse hepatitis. Lipochrome pigment is scanty. No pigment observed in Kupffer cells. Occasional liver cells show bile canaliculi filled with yellow colloid (icterus). Many liver cells show fat droplets. The blood fibrinogen was subnormal during the last weeks of life and we may safely assume a true hepatic insufficiency.

A-982. Cirrhosis and hepatitis—alcoholism—hemoglobin 30 per cent—40 yrs.

Liver—autopsy weight 2700 gm.—laboratory specimen 2580 gm.

Histological specimen—no normal liver tissue anywhere. Remarkably diffuse increase in stroma both in portal region and throughout all parts of lobules. Lobules are full of tiny focal necroses in which wandering cells are numerous. Liver cells are of every type, some large and hydropic, others showing fat, others hyaline necrosis. Pigment is scanty. The regular architecture of the lobule is completely destroyed. New bile ducts are inconspicuous. Blood fibrinogen 0.452 per cent and normal blood clots at autopsy.

A-905. Acute hepatitis—icterus—delirium—soft clots and hemorrhages—hemoglobin 75 per cent—14 yrs.

Liver—autopsy weight 1350 gm.—laboratory specimen 720 gm.

Histological specimen—no normal liver cells, slight portal fibrosis with mononuclears. Central 1/2 of lobules shows extreme fat and even necrosis. Fatty degeneration noted in marginal 1/3 of liver lobule. Here abortive bile duct reaction seen. No pigment. Slight regeneration of liver epithelium, probable hepatic insufficiency.

A-1628. Subacute hepatitis with cirrhosis—icterus—hemoglobin 58 per cent—45 yrs.

Liver—autopsy weight 3590 gm.—laboratory specimen 3170 gm.

Histological specimen—liver lobulation completely destroyed by a diffuse reaction of cells and new formed stroma and bile ducts. There are focal injuries, acute and chronic. The liver cells are much degenerated and show abundant pigment and in places bile canaliculi filled with brown hyaline casts. Pigment is abundant

Pigment is scanty. Occasional focal necroses. The portal stroma is slightly increased and contains many mononuclear cells. Icterus is indicated by some colloid casts in bile canaliculi.

A-42-28. Acute and chronic alcoholism—43 yrs.

Liver—laboratory specimen 1800 gm.

Histological specimen—liver shows extreme fatty degeneration. All liver cells full of large or small fat droplets. Lipochrome noted in some areas.

TABLE 22

Hemoglobin Production Factors in Abnormal Human Liver
Amyloid Liver (Tuberculosis), Fatty Liver (Alcoholism)

Number	Cause of death	Iron content human liver		Liver intake per day		Hemoglobin output per 7 days feeding		
		fresh tissue	daily intake	Human	Control	from Human	from Control	ratio Human to Control
		mg. per cent	mg.	gm.	gm.	gm.	gm.	per cent
A-580	Amyloid	—	—	380	150	49	54	72
A-326	Amyloid	—	—	340	300	43	30	127
A-348	Amyloid	—	—	365	142	46	38	47
A-226	Amyloid	—	—	174	300	12	30	71
A-293	Sl. amyloid	—	—	135	300	32	33	214
A-1359	Alcoholism	5.8	19	380	300	35	50	56
A-42-28	Alcoholism	—	—	260	300	38	30	146
X-510	Alcoholism	—	—	360	225	58	33	110
A-79	Alcoholism	—	—	195	300	43	34	196
X-185	Alcoholism	—	—	193	160	14	30	74
Average.....				278				111

X-510. Acute and chronic alcoholism—40 + yrs.

Liver—laboratory specimen 2520 gm.

Histological specimen—large and small fat droplets in all liver cells. Little lipochrome pigment.

A-79. Acute alcoholism—terminal bronchopneumonia—30 + yrs.

Liver—laboratory specimen 1375 gm.

Histological specimen—liver cells show diffuse fat infiltration with tiny fat droplets. Slight portal cell increase. Much congestion.

X-185. Chronic alcoholism—terminal bronchopneumonia—about 40 yrs.

Liver—laboratory specimen 2700 gm.

X-2353. Suppurative cholangitis—portal cirrhosis—cancer of bile ducts—icterus—38 yrs.

Liver—autopsy weight 1720 gm.—laboratory specimen 1300 gm.

Histological specimen—inflammatory reaction about bile ducts and portal tissue, occasional small abscesses. Central part of lobule may show necrosis or extreme stasis of bile in bile canaliculi. Some liver epithelium fairly well preserved.

Table 25 presents a group of cases in which *severe liver injury* is present and in some instances liver insufficiency is obvious. In this liver material the hemoglobin producing factors are obviously reduced

TABLE 25

*Hemoglobin Production Factors in Abnormal Human Liver
Hepatitis and Functional Liver Insufficiency*

Number	Cause of death	Iron content human liver		Liver intake per day		Hemoglobin output per 7 days feeding		
		fresh tissue	daily intake	Human	Control	from Human	from Control	ratio Human to Control
		mg. per cent	mg.	gm.	gm.	gm.	gm.	per cent
A-956	Hepatitis	1.7	5	290	300	13	53	26
A-982	Hepatitis	1.8	7	360	142	27	38	28
A-905	Hepatitis	—	—	100	150	36	59	92
A-1628	Hepatitis	24.0	109	450	300	74	58	85
A-976	Cirrhosis	2.4	2	100	300	8	30	80
A-709	Cirrhosis	10.9	55	500	142	29	38	22
A-27-28	Cirrhosis	—	—	187	142	26	38	52
X-293	Cirrhosis	—	—	118	300	4	35	29
A-821	Cirrhosis	24.5	35	141	142	13	38	34
X-2353	Cholangitis	1.8	3	186	200	20	62	35
Average.....		9.6	31	243				48

below normal. The average figure is 48 per cent as compared with the normal of 162 per cent. Most of these abnormal livers are below normal weight so that if calculated for normal weight the figures would be still lower.

In one case (A-1628) the iron analyses show a high figure and a daily intake of 109 mg. Fe. This would account for about half the reaction noted in this case which is higher than the average. It is probable that there was blood destruction during the last days of life with excess storage of iron in the phagocytes and hepatic epithelium as noted in sections.

Histological specimen—liver sections show a good deal of diffuse tumor infiltration of the liver lobules about the portal structures. Occasional bile canaliculi show hyaline brown casts. Lipochrome pigment is in evidence. Some Kupffer cells contain a little pigment which stains for iron.

A-367. Cancer of breast—liver metastases—53 yrs.

Liver—laboratory specimen 2400 gm.

Histological specimen—much cancer tissue. Some liver cells normal, but numerous central necroses near cancer areas, also atrophy and lipochrome pigmentation. Bile canaliculi plugged in some areas, probably a good deal of normal liver parenchyma.

TABLE 23

*Hemoglobin Production Factors in Abnormal Human Liver
Liver Carcinoma—Primary and Secondary*

Number	Cause of death	Iron content human liver		Liver intake per day		Hemoglobin output per 7 days feeding		
		fresh tissue	daily intake	Human	Control	from Human	from Control	ratio Human to Control
		mg. per cent	mg.	gm.	gm.	gm.	gm.	per cent
A-512	Liver—Ca.	—	—	733	225	50	30	51
A-54-31	Liver—Ca.	4.7	11	280	300	44	50	94
X-3788	Stomach Ca.	7.1	43	610	300	93	50	91
A-1741	Embryoma	38.7	80	208	300	80	74	157
A-367	Breast Ca.	—	—	343	300	15	41	32
A-129-29	Stomach Ca.	8.4	18	210	300	15	44	48
A-456	Prostate Ca.	13.8	76	555	300	27	34	43
A-359	Gall bladder Ca.	—	—	185	200	47	62	83
Average.....		14.5	46	391				75

A-129-29. Cancer of stomach with liver invasion—icterus—51 yrs.

Liver—laboratory specimen 1500 gm.

Histological specimen—much of the liver section shows tumor tissue or degenerating liver cells. Much fatty degeneration of liver cells found. Some normal liver cells are present. Lipochrome pigment is abundant. Kupffer cells show a yellow pigment.

A-456. Cancer of prostate—extensive liver metastases—78 yrs.

Liver—autopsy weight 4200 gm.—laboratory specimen 3900 gm.

Histological specimen—cancer tissue makes up much of sections. Liver cells show various degrees of atrophy and fatty degeneration plus lipochrome. Large Kupffer cells.

Cirrhosis or hepatitis with severe parenchyma injury and signs of hepatic insufficiency give low values (Table 25) in biological assay—48 per cent compared to normal human 162 per cent. It may be surprising that the liver cells hold so tenaciously to these unknown factors influencing hemoglobin production but obviously only in severe liver injury is this concentration seriously reduced.

Another case (A-1741) Table 23, shows a high iron analysis and this iron intake undoubtedly is a factor in the production of hemoglobin. This will in a measure offset the "dilution" of the liver specimen by tumor tissue but in this instance the final figure would be close to 150 per cent or normal.

Table 24. *Diagnosis and histological description of liver.*

A-1589. Liver cirrhosis (hob-nail)—ascites—hemoglobin 75 per cent—74 yrs.
Liver—autopsy weight 1170 gm.—laboratory specimen 1000 gm.
Histological specimen—a typical annular fibrosis involving portal structures but not the islands of preserved liver cells which in some areas show fat but elsewhere may be normal. Very little pigment anywhere. Occasional focal necroses. Kupffer cells inconspicuous. Bile ducts inconspicuous.

A-1468. Liver cirrhosis (hob-nail)—ascites—pneumonia—61 yrs.
Liver—autopsy weight 1560 gm.—laboratory specimen 1480 gm.
Histological specimen—usual picture of hepatic cirrhosis with much scar tissue which contains numerous mononuclears. Many liver cells normal. New formed bile ducts not conspicuous. Post mortem autolysis is well marked. Large bile ducts normal. Pigment scanty. Kupffer cells normal.

A-615. Liver cirrhosis (hob-nail)—nephrosis—syphilis—hemoglobin 79 per cent—49 yrs.
Liver—autopsy weight 1450 gm.—laboratory specimen 1300 gm.
Histological specimen—considerable portal fibrosis with mononuclears and some new bile ducts. Liver lobules distorted but parenchyma looks normal except for cloudy swelling and little fat. No pigment. Kupffer cells normal.

A-1114. Liver cirrhosis (hob-nail)—icterus—hemoglobin 35 per cent—56 yrs.
Liver—autopsy weight 1950 gm.—laboratory specimen 1740 gm.
Histological specimen—typical annular periportal cirrhosis. The new scar tissue is full of mononuclears. Bile duct buds are not numerous. The hepatic epithelium is relatively normal except for icterus. Bile canaliculi conspicuous and filled with dark colloid. Kupffer cells contain a little pigment. Patient had been bleeding into gastro-intestinal tract, kidneys and body tissues, the last few weeks of life and this caused anemia. It is likely that some antistubstance was present in blood and responsible for the bleeding. Blood fibrinogen normal. Hepatic insufficiency seems unlikely.

A-788. Liver cirrhosis (hob-nail—alcoholic)—terminal pneumonia—hemoglobin 90 per cent—44 yrs.
Liver—laboratory specimen 1275 gm.
Histological specimen—much annular portal fibrosis, many lobules in part de-

used. This also explains the well known fact that recovery and hemoglobin reconstruction are so spectacular in pernicious anemia when this missing factor is supplied—a large surplus of all other needed building materials being at hand. By contrast the recovery from secondary anemia is much slower as all these building materials must be produced within the body largely from food intake and this may consume a good deal of time.

Richter, Ivy and Kim (2) have made interesting observations relating to the liver in pernicious anemia. They find that the liver in an untreated case of pernicious anemia contained no factors which are abundant in the normal liver and promote a remission in human cases of pernicious anemia. Also the treated pernicious anemia case contains in its liver the material which promotes a remission in another case of pernicious anemia.

Aplastic anemia is due to lack of red bone marrow to furnish needed red cells. What causes the red marrow to shrivel to a mere remnant is not known. In this disease it is observed (Table 32) that the liver stores excess of hemoglobin building material and this is not surprising as there is no outlet for this material.

In *secondary anemia* we expected to find that these hemoglobin factors were much reduced if not almost completely exhausted. Much to our surprise it was found (Table 33) that in *anemia due to loss of blood* the liver contained a low normal concentration of these hemoglobin producing factors. This is true for man (Table 33) and for the horse (unpublished data) and therefore probably applies to other warm blooded animals.

Secondary anemia due to blood destruction within the body may present a different picture. The last case in Table 33 illustrates this point but more observations are needed. It is not surprising that the liver stores iron and hemoglobin producing factors when red cells are being destroyed in the body.

Leukemia is almost always associated with more or less anemia, the terminal hemoglobin figures being about 30–40 per cent of normal. Sometimes blood transfusions with or without bleeding will modify the picture. When one examines the bone marrow it is not difficult to visualize the anemia as due to marrow disturbance. The red cell producing chain of cells in the marrow is crowded by the great mass

Liver—autopsy weight 930 gm.—laboratory specimen 770 gm.

Histological specimen—much portal scarring with mononuclears and new formed ducts and distortion of lobules, much liver parenchyma is normal but for much fatty degeneration and scattered large and small liver cell necroses. Kupffer cells large and contain no pigment.

A-789. Liver cirrhosis—lobar pneumonia—hemoglobin 85 per cent—45 yrs.

Liver—laboratory specimen 2775 gm.

Histological specimen—little portal fibrosis but many mononuclears and few polymorphonuclears. Occasional focal necroses in liver cells close to these portal areas. No pigment in liver cells but granular degeneration and little fat. Kupffer cells normal. Most of section shows normal liver cells.

A-29-77. Liver cirrhosis (hob-nail)—icterus.

Liver—laboratory specimen 940 gm.

Histological specimen—much scar tissue, possibly 1/3 of section, full of lymphocytes and new bile ducts, lipochrome abundant. Liver parenchyma hypertrophied, some diffuse fatty degeneration. Few necroses, some lobules show icterus plugs in canaliculi, some not. No hepatic insufficiency.

A-28-168. Liver cirrhosis (alcoholic)—terminal bronchopneumonia.

Liver—autopsy weight 2600 gm.—laboratory specimen 2590 gm.

Histological specimen—much post mortem change. Scars abundant and coarse, perhaps replacing 1/4 entire section, much bile duct proliferation. Liver cells show much fat, bile canaliculi show numerous colloid plugs. Lipochrome abundant.

A-27-160. Liver cirrhosis—cystitis—pyelonephritis.

Liver—laboratory specimen 910 gm.

Histological specimen—coarse scars, liver parenchyma looks well and makes up 4/5 of sections. Infectious biliary type? No bile duct regeneration, mononuclears found in scars. Few indefinite central necroses and little fat. No icterus. No hepatic insufficiency.

A-51-27. Liver cirrhosis.

Liver—laboratory specimen 750 gm.

Histological specimen—slight periportal fibrosis and clusters of mononuclears, like biliary cirrhosis in early stage. Few colloid bile plugs in canaliculi. Many large and small fat droplets in liver cells, few necroses of 1-2 cells. Few polymorphonuclears. Lipochrome inconspicuous.

X-334. Liver cirrhosis—syphilis—alcoholism—cerebral hemorrhage—42 yrs.

Liver—autopsy weight 2450 gm.—laboratory specimen 2340 gm.

Histological specimen—liver presents the usual picture of a diffuse cirrhosis. The

Histological specimen—the liver cells in general stain well and contain a finely granular yellow pigment which in part gives a positive stain for iron. This pigment is more conspicuous in the central part of the lobules. In a few lobules are noted small central necroses. The Kupffer cells show very little pigment.

X-2479. Pernicious anemia—pyelonephritis—no information about liver therapy—75 yrs.

Liver—autopsy weight 1500 gm.—laboratory specimen 1350 gm.

Histological specimen—central necroses are conspicuous and occupy perhaps $1/5$ of the liver lobule. The liver cells in the peripheral $1/2$ of the lobule are normal. There is abundant fine granular yellow pigment giving a strong iron stain in these liver cells. Kupffer cells contain no pigment.

A-1472. Pernicious anemia—subdural hemorrhage—hemoglobin 30–40 per cent—68 yrs.

Liver—autopsy weight 1260 gm.—laboratory specimen 1100 gm.

Histological specimen—liver cells almost normal. There is some finely granular yellow pigment but very little of this gives a stain for iron. Kupffer cells contain fine pigment granules which stain sharply for iron. This case had liver extract for 10 days with the usual blood improvement and this explains some of the pigment lack in liver and kidney.

A-425. Pernicious anemia—senility—hemoglobin 30 per cent—78 yrs.

Patient in Hospital May 25, 1927—hemoglobin 25 per cent—and improved on treatment. Left Hospital August 12, 1927—hemoglobin 65 per cent. Readmitted in moribund state and died in 24 hrs.—no therapy for over 1 mo.

Liver—laboratory specimen 1050 gm.

Histological specimen—liver cells in general normal. Few large fat droplets in periportal liver cells, some atrophy, lipochrome moderate. Kupffer cells numerous and full of iron-containing pigment.

A-1122. Pernicious anemia—hemoglobin 30–80 per cent—pulmonary emboli—75 yrs.

Liver—autopsy weight 1000 gm.—laboratory specimen 930 gm.

Histological specimen—liver cells in general are normal except for fine granular yellow pigment in their protoplasm. Kupffer cells show similar pigment grains in small amount. Some of this pigment gives a stain for iron. A few liver cells show fat droplets. This case had been benefited by liver therapy and at death the hemoglobin was 80 per cent.

A-1173. Pernicious anemia—hemoglobin 25 per cent—bronchopneumonia—no liver therapy during last 6 months of life—24 hrs. in hospital—74 yrs.

Liver—laboratory specimen 1130 gm.

A-205. Liver cirrhosis (slight)—organizing pneumonia—43 yrs.
Liver—autopsy weight 2540 gm.—laboratory specimen 2050 gm.
Histological specimen—moderate to slight portal fibrosis, few new bile ducts.
Good deal of fat in central 3/5 of lobule, some degeneration with lipochrome pigment deposit in liver cells.

A-730. Liver cirrhosis (slight)—pneumonia lobar—46 yrs.
Liver—autopsy weight 1500 gm.—laboratory specimen 1400 gm.
Histological specimen—slight annular fibrosis with no new bile duct proliferation.
Many mononuclears, slight lobule distortion, little lipochrome. Liver parenchyma looks normal but for little fat. Occasional tiny hyaline necroses.

A-1024. Liver cirrhosis—icterus—carcinoma pancreas—53 yrs.
Liver—autopsy weight 1550 gm.—laboratory specimen 1500 gm.
Histological specimen—there is moderate portal cirrhosis and duct proliferation.
The liver cells are well preserved and approximate a normal appearance. There are no necroses. Bile canaliculi often contain yellow colloid. Pigment is abundant in hepatic epithelium and Kupffer cells.

Table 24 shows a considerable variety of *liver cirrhosis* and hepatitis without any evidence of hepatic insufficiency. These cases died from terminal infections, hemorrhage or alcoholism and presented more or less severe passive congestion of the abdominal viscera with all grades of icterus. It is obvious from a glance at Table 24 that the concentration of the hemoglobin production factors is not abnormal in these liver specimens. There are considerable individual variations but from the average of 164 per cent we see the low values rarely below 100 per cent and the high values rarely above 200 per cent.

Case A-1114, Table 24, presents the lowest value for the hemoglobin production factors and there are some points which indicate a possible hepatic insufficiency (see Table 25). A-1114 showed bleeding into the intestinal tract and body tissues but a normal fibrinogen. The liver cells did not appear seriously abnormal histologically. It seemed safest to place this case in the group of cirrhotoses without convincing evidence of severe liver injury and insufficiency.

The iron analyses show an average value which is a trifle below the normal averages. The lowest values appear in association with secondary anemia—compare Table 33 (Paper III).

Icterus was present in varying degrees in this group of cases and we see no reason to suspect that bile pigment stasis influences the concentration of hemoglobin producing factors in the liver cells.

A-371 show an average of 33 mg. per cent or about 3 times normal and the liver values for hemoglobin production factors are high. The lowest value in hemoglobin production factors appears in Case X-2479 (Table 31) which was complicated by a pyelonephritis which caused death. It is probable that this acute condition would reduce somewhat the content of hemoglobin producing factors in the liver—compare Tables 3 and 4 (Paper I).

In a comparative study of the cases in Table 31 we must keep in mind that all these cases show senile atrophy and the normal mean for this type of case is given in Table 2 (Paper I) as 117 per cent which contrasts with 218 per cent in Table 31. If we exclude the unusual figure in the first case, the average value for hemoglobin producing factors is 182 per cent. Even if we choose to explain some of this difference in potency on the basis of the contained iron we have an excess coming from unknown factors stored within the liver parenchyma cells. We assume that this excess represents building stones which are suitable for hemoglobin construction in the normal body but have no outlet in primary anemia.

Table 32. *Diagnosis and histological description of liver.*

A-376. Aplastic anemia—many transfusions—bleeding—30 yrs.

Liver—laboratory specimen 1750 gm.

Histological specimen—swollen liver cells, some central liver cells are injured, few necroses. Portal liver cells full of yellow pigment. Few Kupffer cells show pigment. Both liver cells and Kupffer cells show iron-containing pigment.

A-1555. Aplastic anemia—purpura—transfusions—hemoglobin 40–60 per cent—57 yrs.

Liver—autopsy weight 1490 gm.—laboratory specimen 1300 gm.

Histological specimen—liver cells show many large and small fat droplets. They contain much yellow granular pigment much of which gives a stain for iron. Kupffer cells are large and contain iron staining pigment (transfusions). Bile ducts and stroma normal.

A-924. Aplastic anemia—hemoglobin 20 per cent—40 yrs.

Liver—laboratory specimen 1640 gm.

Histological specimen—the liver cells in general are normal and contain a little lipochrome pigment. There are scattered small central necroses. A moderate degree of fat deposit in liver cells in the mid zone is noted. No iron staining pigment found.

in phagocytes in the stroma. Pigment is seen in the epithelium of the new formed bile ducts. Mononuclears and polymorphonuclears are abundant in all parts of the section. This liver appears insufficient yet the blood fibrinogen was normal. The pigment in phagocytes gives an iron stain—only a part of the pigment in hepatic epithelium gives an iron stain.

A-976. Liver cirrhosis—alcoholism—syphilis—hemoglobin 100 per cent—41 yrs. Liver—autopsy weight 800 gm.—laboratory specimen 700 gm.

Histological specimen—a coarse annular cirrhosis of moderate grade. The stroma between lobules is dense and filled with mononuclear cells and bile duct sprouts. Much liver parenchyma appears but slightly injured but there is much fatty degeneration and occasional focal necroses. The fibrinogen was subnormal (0.21 per cent) so that the liver was probably functionally subnormal.

A-709. Liver cirrhosis with fat—alcoholism—icterus—syphilis—soft blood clots—hemoglobin 92 per cent—54 yrs.

Liver—autopsy weight 5280 gm.—laboratory specimen 3500 gm.

Histological specimen—moderate annular portal fibrosis with some bile duct proliferation and scanty mononuclears. Pigment abundant in some cells; bile canaliculi are distended with colloid. Liver cells much injured, great amounts of fat. Some central necroses, many focal necroses. Hepatic insufficiency? The blood clots in this case were quite soft.

A-27-28. Liver cirrhosis—ascites—bronchopneumonia—46 yrs.

Liver—autopsy weight 1530 gm.—laboratory specimen 1460 gm.

Histological specimen—more than 1/2 of section is new bile ducts and scar tissue. Much icterus. Many leucocytes. Islands of liver parenchyma make up less than 1/2 of the section. Much necrosis of remaining liver cells. Hepatic insufficiency?

X-293. Liver cirrhosis and icterus.

Liver—autopsy weight 840 gm.—laboratory specimen 825 gm.

Histological specimen—portal fibrosis and duct regeneration conspicuous, possibly 3/5 of liver parenchyma in fair condition but for icteric casts in bile canaliculi. Leucocytes and polymorphonuclears in portal stroma.

A-S21. Liver cirrhosis (hob-nail)—ascites—icterus—bronchopneumonia—hemoglobin 40 per cent—81 yrs.

Liver—autopsy weight 1060 gm.—laboratory specimen 990 gm.

Histological specimen—moderate portal fibrosis with many mononuclears; here too black granular pigment in phagocytes (malarial or coal). Iron stain negative in all cells. Some deformity of liver lobules, much of section shows hypertrophied liver lobules with good epithelium, some granular degeneration and post mortem change. Giant nuclei, much lipochrome.

Liver—autopsy weight 1300 gm.—laboratory specimen 1030 gm.
Histological specimen—necrosis of central type involving 1/5 or less of liver parenchyma. Fat droplets numerous in mid zone. The liver cells in the peripheral half of lobules appear normal. Lipochrome pigment scanty.

A-1333. Anemia secondary to bladder carcinoma—hemoglobin 15 per cent—51 yrs.

Liver—autopsy weight 1220 gm.—laboratory specimen 1030 gm.
Histological specimen—liver cells in general normal—no pigment seen. Fatty degeneration is found in the centers of lobules. The portal tissue is increased and filled with mononuclears. The casts in some of the bile canaliculi are very dark and of some duration, probably related to the cholelithiasis and an earlier cholangitis.

A-1461. Anemia secondary to cancer of stomach—cirrhosis—hemoglobin 40 per cent—47 yrs.

Liver—autopsy weight 1800 gm.—laboratory specimen 1700 gm.
Histological specimen—liver lobules are relatively normal and hepatic epithelium not disturbed and practically normal but for some fatty degeneration. An annular type of portal cirrhosis is observed but the connective tissue is not dense and the distortion but slight. This scar tissue contains many mononuclears but few bile duct sprouts. No pigment is observed anywhere.

A-1971. Anemia secondary to carcinoma of stomach—hemoglobin 50 per cent—64 yrs.

Liver—autopsy weight 1200 gm.—laboratory specimen 1175 gm.
Histological specimen—liver cells in peripheral half of lobule show large fat vacuoles. Liver cells in center of lobule normal. Lipochrome scanty. Portal tissue slightly increased and infiltrated with mononuclears.

A-1375. Anemia secondary to carcinoma of stomach—60 yrs.

Liver—autopsy weight 1750 gm.—laboratory specimen 1600 gm.
Histological specimen—the liver cells are practically normal except for rather abundant lipochrome pigment. Kupffer cells normal.

A-1372. Anemia secondary to carcinoma of kidney—hemoglobin 50 per cent—57 yrs.

Liver—autopsy weight 2160 gm.—laboratory specimen 2100 gm.
Histological specimen—liver lobules show central congestion and cell atrophy with abundant lipochrome pigment. Marginal half of lobules essentially normal.

A-1081. Anemia secondary to hypernephroma—hemoglobin 50 per cent—60 yrs.

Liver—autopsy weight 1850 gm.—laboratory specimen 1540 gm.
Histological specimen—practically normal hepatic epithelium. Lipochrome pig-

Cases of severe liver injury with anemia give the lowest values by biological assay. The concentration of hemoglobin producing factors may be less than one-fourth of normal. This invites speculation. We may say that anemia alone does not appreciably reduce these hemoglobin production factors (Table 33, Paper III). It may be argued that the anemia is due to infections or tissue or intestinal bleeding some time before death. This may in fact explain much of the anemia but we would suggest that a factor may be introduced relating to liver function. There is some evidence to indicate that the liver participates in active fashion in the preparation of parent substances for hemoglobin production. Given a serious liver dysfunction in these cases we have another cause for the anemia.

SUMMARY

Biological assay of human liver material shows various concentrations of hemoglobin producing material in various diseased states.

Cardiac congestion with red atrophy of the liver shows values (Table 21) somewhat below the human normal—94 per cent compared to 162 per cent. It is probable that this is due to atrophy and destruction of the liver cells in the centers of the liver lobules. We believe the remaining liver cells have a normal content of hemoglobin producing factors.

Amyloid (tuberculosis) and fatty (alcoholic) degeneration show (Table 22) subnormal values—111 per cent compared to 162 per cent. If we allow for the "dilution" of liver cell weight by the amyloid and fat we observe normal values for the concentration of hemoglobin producing factors.

Carcinoma growth in the liver whether primary or secondary shows net figures (Table 23) which are low—75 per cent compared to normal 162 per cent. It appears that cancer tissue, even tumor cells originating from hepatic epithelium, contains no hemoglobin producing factors. If we allow for the "dilution" of the remaining liver cells by the cancer tissue the figures are within normal limits.

Cirrhosis and hepatitis (Table 24) with no signs of true hepatic insufficiency or severe hepatic injury but death supervening from secondary infection or hemorrhage will give average normal values—164 per cent compared to normal human of 162 per cent. Icterus is not a factor.

Table 33 shows 9 cases of severe secondary anemia due to loss of blood and one case due to blood destruction within the body. Biochemical assay of the hemoglobin production factors in these 10 cases show low normal values (135 per cent) and if we exclude the single case of hemolytic anemia, the average for the 9 cases of anemia due to loss of blood from the body is 126 per cent. This shows how tenacious the liver cell holds to these hemoglobin production factors in the face of a long continued severe anemia due to loss of blood. In contrast the *iron analyses* show that the normal iron store is considerably reduced by this type of anemia. The normal iron value (100 mg. per cent) contrasts with 5.3 mg. per cent, the average for 9 cases of secondary anemia (Table 33) due to loss of blood. These analyses are not quite accurate because of blood contained in the capillaries and do not measure accurately the iron contained within the liver cells. They are in general accord with accurate figures obtained from blood free liver tissue in dogs (1) which show that about 10 weeks of severe anemia due to bleeding will reduce liver parenchyma iron to an irreducible minimum of 4-5 mg. per cent.

In the case of hemolytic anemia due to rheumatism—A-1618, Table 33 as expected shows a high figure for iron (23 mg. per cent) and for the content of hemoglobin producing factors 217 per cent. The red blood cells were destroyed within the body and this salvaged material stored in the liver.

Table 34. *Diagnosis and histological description of liver.*

9. Acute leukemia—hemoglobin 80 to 35 per cent 3 wks.—46 yrs.
 Autopsy weight 1510 gm.—laboratory specimen 1320 gm.
 Histological specimen—liver sections show typical leukemic infiltration. Liver shows some atrophy and a few small fat droplets—also some lipochrome pigment. Kupffer cells are large but show no pigment. No iron staining pigment detected.

Acute leukemia—hemoglobin 30-40 per cent—terminal bronchopneumonia—56 yrs.
 Autopsy weight 1950 gm.—laboratory specimen 1675 gm.
 Histological specimen—much degeneration of liver cells which are very granular and swollen. Small fat droplets numerous. Central atrophy plus lipochrome. Scattered large hyaline necroses of liver parenchyma.

HEMOGLOBIN PRODUCTION FACTORS IN THE HUMAN LIVER

III. ANEMIAS—PRIMARY, APLASTIC AND SECONDARY—LEUKEMIAS

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The various anemias and leukemias with consequent anemia present material of especial interest for a study of this character. Biological assay of this human liver material shows high values for primary and aplastic anemias but low normal values for the secondary anemias due to loss of blood as well as for leukemias. A number of interesting points emerge from a study of this material and call for discussion.

Pernicious anemia at the present time is being subjected to intensive scrutiny by the physiologist, chemist and clinician and considerable progress is being made toward a more complete understanding of this disease. The findings tabulated below will be surprising or not, depending upon the conception which the reader may hold relating to the pathogenesis of pernicious anemia. On the basis of anatomical findings it was suggested (3) more than 10 years ago that pernicious anemia could be explained best as due to some lack of unknown material which might be responsible for red cell stroma production. It was pointed out that everywhere there was a large excess of pigment material in the red cells, body fluids and tissues, but this pigment material could not be used because of this lack of some essential factor. At the time this suggestion was made, the general belief in some unknown toxic substance responsible for the anemia was almost universal.

The tabulated data (Table 31) below give support to this hypothesis and show a definite excess of hemoglobin building factors stored in the liver in pernicious anemia in spite of the fact that death may be due to lack of red cells and hemoglobin. Surely there is a maximal stimulus for the production of red cells and hemoglobin but some essential keystone is missing and the other building stones cannot be

A-377. Acute leukemia—hemoglobin 70 per cent—90,000–150,000 white blood cells—56 yrs.

Liver—laboratory specimen 2650 gm.

Histological specimen—liver cells swollen, very little fat, central liver cells show much yellow pigment (lipochrome). No iron staining granules. Lymphocytes abundant especially about the portal tissue. Kupffer cells large and some show little pigment but red blood cell and white blood cell inclusions.

A-1963. Acute myeloid leukemia—15 per cent hemoglobin—48 yrs.

Liver—autopsy weight 1750 gm.—laboratory specimen 1560 gm.

Histological specimen—liver lobules contain many white cells in the capillaries and in the periportal stroma but there is no conspicuous infiltration. There are scattered small hyaline necroses. The liver cells contain some fat droplets and lipochrome pigment. Kupffer cells are large and contain some yellow granular pigment which stains for iron. Fine pigment grains in the liver cells also give a stain for iron.

A-1349. Leukemia—myeloid—hemoglobin 35 per cent—65 yrs.

Liver—autopsy weight 1775 gm.—laboratory specimen 1550 gm.

Histological specimen—central hyaline necrosis is conspicuous and possibly involves 1/10 of the liver cells. Cell infiltration of the lobules is not conspicuous. Pigment within the liver cells is abundant; that in the center of the lobules gives no iron stain (lipochrome) but some pigment grains in the periphery of the lobules do give a faint iron stain. Kupffer cells are conspicuous and some contain pigment which gives no iron stain.

A-316. Myeloid leukemia—hemoglobin 60 per cent down to 40 per cent—bleeding—71 yrs.

Liver—laboratory specimen 1980 gm.

Histological specimen—great numbers of myelocytes everywhere, perhaps making up 1/4 of the tissue or more. Liver cells are granular and rich in pigment which gives a strong stain for iron. No fat droplets. Kupffer cells not conspicuous and contain no pigment.

A-1828. Leukemia chronic lymphoid—hemoglobin 30 per cent—59 yrs.

Liver—autopsy weight 2700 gm.—laboratory specimen 2480 gm.

Histological specimen—there is only a slight leukemic infiltration of the portal tissue and liver lobules. There is abundant lipochrome pigment in the liver cells especially in the central part of the lobule. Kupffer cells show no pigment.

A-1943. Chronic lymphatic leukemia—hemoglobin 55 per cent—66 yrs.

Liver—autopsy weight 3360 gm.—laboratory specimen 3180 gm.

Histological specimen—the liver lobules show extreme infiltration with lympho-

of white cells and its function probably impaired. Biological assay shows that the leukemic liver and the liver of secondary anemia stand about on a par, estimated per gram gross liver weight. There is no evidence that these abnormal white cells which infiltrate the leukemic liver and increase its weight contribute any conspicuous amount of hemoglobin producing factors. If we say that these white cells "dilute" the potency of the liver cells, the figures for the leukemic liver potency would rise to normal.

The *iron analyses* deserve particular attention. The cases of secondary anemia due to bleeding show very low values (5.3 mg. per cent) as is to be expected and this is in harmony with the experiments in animals recently reported (1). Leukemia shows about the normal iron content but if we allow for the large size of the liver the actual liver parenchyma may contain a little more iron than the normal human control. The iron analyses for primary and aplastic anemias are very high (5-10 times normal) and we may believe this is a storage of material of use for hemoglobin construction but in these diseases there is no outlet for this material in normal hemoglobin production. The figures are higher in aplastic anemia than in primary anemia which is evidence against hemoglobin destruction being a large factor in primary anemia. The iron concentration in pernicious anemia drops rapidly with specific liver therapy.

Table 31. *Diagnosis and histological description of liver.*

A-371. Pernicious anemia—typical—hemoglobin 15-20 per cent—77 yrs.

Liver—autopsy weight 1050 gm.—laboratory specimen 900 gm.

Histological specimen—typical untreated pernicious anemia—yellow pigment very abundant in liver cells and Kupffer cells (much of this pigment contains iron). A little liver cell atrophy, no fat, slight portal fibrosis.

A-1800. Pernicious anemia relapse—hemoglobin 20 per cent—60 yrs.

Liver—autopsy weight 1850 gm.—laboratory specimen 1760 gm.

Histological specimen—autopsy typical of pernicious anemia without liver treatment. Liver shows the usual pigment in gross and histologically. Iron staining pigment is abundant in the liver cells and also Kupffer cells. No liver necroses.

A-1045. Pernicious anemia—hemoglobin 30 per cent—pneumonia—during last 3 months had been taking liver irregularly—51 yrs.

Liver—autopsy weight 2150 gm.—laboratory specimen 1920 gm.

numbers of parent or undifferentiated cells in the blood, marrow and viscera. Two cases of chronic lymphoid leukemia show low values by biological assay (57 and 84 per cent). We have no explanation for these low figures.

The anemia in leukemia would seem to depend upon marrow insufficiency due to encroachment of the white cells on the red cell chain. There is little or no evidence for blood destruction as the iron analyses are close to normal.

Table 35. *Diagnosis and histological description of liver.*

A-1367. Familial anemia (Cooley)—siderosis of viscera—hemoglobin 50 per cent—5 yrs.

Liver—autopsy weight 860 gm.—laboratory specimen 740 gm.

Histological specimen—the liver cells show large deposits of iron staining pigment. Kupffer cells contain much pigment and some stains for iron. The liver cells are relatively normal but for the pigment. There is a little central fatty degeneration.

A-1819. Hemochromatosis—cancer of stomach—hemoglobin 78 per cent—57 yrs.

Liver—autopsy weight 2170 gm.—laboratory specimen 1980 gm.

Histological specimen—typical picture of moderately advanced case of hemochromatosis with annular cirrhosis. There is little new bile duct proliferation. Iron staining pigment is abundant in liver epithelium and especially in phagocytes in stroma about the portal areas. Kupffer cells are rich in the same pigment. There are calcium deposits in portal stroma.

A-526. Peculiar leukemia with marrow aplasia—liver pigment rich in iron—44 yrs.

Red blood cells 640,000; white blood cells 1600; hemoglobin 10 per cent. Differential 80 per cent lymphocytes. Platelets numerous.

Liver—laboratory specimen 2150 gm.

Histological specimen—central necroses involve about 1/4–1/3 of liver lobules. Many mononuclears here. Much pigment in liver cells and some gives an iron stain. A little fat in mid zone.

A-1785. Meningitis—late chloroform poisoning with extreme liver injury causing death—12 yrs.

Liver—autopsy weight 1560 gm.—laboratory specimen 1460 gm.

Histological specimen—liver sections show not a single normal liver cell. There is much necrosis of the central liver cells but remaining liver cells may show a pale nucleus and a mass of fat droplets replacing the protoplasm. Typical picture of

Histological specimen—liver cells in general are normal. There is abundant lipochrome pigment but no iron staining pigment within the liver epithelium. There is iron staining pigment in the Kupffer cells. This lack of pigment is probably due to preceding liver therapy and blood improvement 10 mos. before death.

Table 31 contains 8 cases of primary pernicious anemia in which various amounts of liver therapy had been given during life. The first case (A-371) is of especial significance as this man had had no liver therapy at any time and presented the classical picture of pernicious anemia which is very rarely seen today. The iron analyses show maximal figures (162 mg. per cent) as does the biological assay for liver

TABLE 31
Hemoglobin Production Factors in Abnormal Human Liver
Pernicious Anemia

Number	Cause of death	Iron content human liver		Liver intake per day		Hemoglobin output per 7 days feeding		
		fresh tissue	daily intake	Human	Control	from Human	from Control	ratio Human to Control
		mg. per cent	mg.	gm.	gm.	gm.	gm.	per cent
A-371	No therapy	162.0	208	129	300	63	35	420
A-1800	No therapy	36.7	92	250	300	97	74	157
A-1045	Sl. therapy	47.3	130	290	300	112	56	208
X-2479	Nephritis	36.5	70	190	105	56	30	104
A-1472	Sl. therapy	17.5	27	158	300	52	50	200
A-425	Sl. therapy	34.8	52	150	300	45	34	265
A-1122	Embolism	24.6	33	130	300	25	30	192
A-1173	No therapy	—	—	160	300	37	46	148
Average.....		51.3	87	182				218

potency (420 per cent) and this great excess of iron is a factor in this high figure for hemoglobin production. In the various tables given in these papers, a biological assay for liver potency above 300 per cent is unusual and figures above 400 per cent have been observed only twice—one case of thyrotoxicosis (Table 4, Paper I) and this case of primary anemia.

No liver therapy in the final relapse is recorded in 2 other cases (A-1800 and A-1173). In these cases liver therapy had been given in earlier periods but not during the last few weeks or months preceding death from a relapse. The liver analyses for iron exclusive of Case

daily iron intake and half the hemoglobin output. Here is a conspicuous dissociation of the iron factors and the hemoglobin production factors in two livers which have many similarities.

The third case (A-526) presented diagnostic difficulties. At first it was classed as a chronic lymphatic leukemia of atypical type but subsequently there was evidence of marrow aplasia which was confirmed at autopsy. The biological assay of hemoglobin producing factors (65 per cent) would put it in the group of chronic leukemias whereas the iron analyses (68 mg. per cent) suggest aplastic anemia. We prefer not to attempt to classify this case at the present time.

Chloroform poisoning (4th case, A-1785) is unusual in many ways. We expected a very low value for the hemoglobin producing factors as the patient died from chloroform injury of liver with low fibrinogen, bleeding and all the essential features of this condition. Moreover the liver shows histological injury of extreme grade. Yet there is abundant material in the abnormal liver tissue from which the normal amount of hemoglobin is produced (159 per cent). It is suggested that these unknown factors remain even in these dead and injured cells as the process was acute and the injured cells have not yet autolyzed and been removed as follows repair in chloroform poisoning. The acuteness of the injury differentiates this type of case from the other type of hepatic insufficiency and liver injury given in Table 25 (Paper II).

Eclampsia presents an unusual observation (A-62, Table 35). Only one other case (Table 21, Paper II) with severe passive congestion gives zero value for hemoglobin production factors. We cannot correlate these two observations but subsequent findings in eclampsia would be of interest.

The last case (A-1261, Table 35) is of considerable interest because it suggests that lactation may reduce the store of hemoglobin producing factors in the normal liver. There is some evidence that material which can be used to build hemoglobin in an emergency (anemia) may be used to build up and replace plasma protein or tissue protein when needed. The production of milk protein might come into this same group. We have long suspected that it might be possible to show experimentally that building materials coming to the liver might be used for one important product (hemoglobin) at one time and again for

A-188. Aplastic anemia—terminal infection—4½ yrs.

Liver—autopsy weight 910 gm.—laboratory specimen 900 gm.

Histological specimen—liver cells normal. Lipochrome in liver cells. Large Kupffer cells are numerous and some contain iron staining pigment.

Table 32 shows 4 typical cases of aplastic anemia. Many transfusions had been given in the first 2 cases and this may explain in part the very high values for iron (78 and 105 mg. per cent). These high iron values will explain a part of the large excess of hemoglobin production observed in these cases. Moreover we should remember that this group represents a lower age period and consequently the normal base line should be 160 per cent (compare Table 1) for accurate control. When we make these allowances the aplastic anemias are not as much

TABLE 32
Aplastic Anemia

Number	Cause of death	Iron content human liver		Liver intake per day		Hemoglobin output per 7 days feeding		
		fresh tissue	daily intake	Human	Control	from Human	from Control	ratio Human to Control
		mg. per cent	mg.	gm.	gm.	gm.	gm.	per cent
A-376	Bleeding	78.0	196	250	300	46	33	164
A-1555	Bleeding	105.0	195	185	300	98	72	213
A-924	Anemia	26.8	63	230	300	58	44	171
A-188	Infection	—	—	120	225	43	30	269
Average.		69.9	151	196				201

above normal by biological assay of the hemoglobin producing factors as is true for pernicious anemia. It may be argued that aplastic anemia does not last as long as primary anemia and that the liver therefore does not accumulate as large a surplus store as is true for pernicious anemia. In both cases there is no outlet for this hemoglobin production material—in primary anemia due to deficiency factors and in aplastic anemia due to lack of red marrow parent cells.

Table 33. *Diagnosis and histological description of liver.*

A-969. Anemia, secondary to carcinoma of cervix with bleeding—hemoglobin 25 per cent—43 yrs.

ment is fairly abundant, particularly in the central portion of the lobules. Kupffer cells show no pigment.

A-1192. Anemia secondary to carcinoma of prostate—hemoglobin 40 per cent—uremia—68 yrs.

Liver—autopsy weight 1680 gm.—laboratory specimen 1650 gm.

Histological specimen—liver cells in general are normal. Lipochrome pigment noted in center of lobules where also liver cells show a few fat droplets. Kupffer cells normal.

A-1625. Anemia secondary to gastric carcinoma—hemoglobin 58 per cent—61 yrs.

Liver—autopsy weight 1550 gm.—laboratory specimen 1480 gm.

TABLE 33
Secondary Anemia

Number	Cause of death	Iron content human liver		Liver intake per day		Hemoglobin output per 7 days feeding		
		fresh tissue	daily intake	Human	Control	from Human	from Control	ratio Human to Control
		mg. per cent	mg.	gm.	gm.	gm.	gm.	per cent
A-969	Ca. cervix	2.9	4	150	300	26	52	100
A-1333	Ca. bladder	3.3	5	145	300	35	52	140
A-1461	Ca. stomach	4.0	10	240	300	30	38	100
A-1971	Ca. stomach	4.6	8	167	300	46	58	144
A-1375	Ca. stomach	2.8	6	225	300	23	45	68
A-1372	Ca. kidney	7.7	23	300	300	53	35	152
A-1081	Hypernephroma	—	—	220	300	52	35	200
A-1192	Ca. prostate	10.6	25	220	300	22	30	100
A-1625	Ca. stomach	6.7	14	210	300	44	49	129
A-1618	Endocarditis	23.2	29	125	300	65	72	217
Average.....		7.3	14	200				135

Histological specimen—liver lobules show a central hyaline necrosis involving perhaps 10-20 per cent of the hepatic epithelium. Liver cells elsewhere show a little fat and lipochrome pigment. Kupffer cells, stroma and bile ducts normal.

A-1618. Subacute rheumatic endocarditis—hemoglobin 35 per cent—34 yrs.

Liver—autopsy weight 900 gm.—laboratory specimen 880 gm.

Histological specimen—liver cells show atrophy and many fat droplets but very little pigment. Kupffer cells show large amounts of pigment and are greatly enlarged. This pigment gives heavy stain for iron. The stroma and bile ducts normal. Anemia due to red blood cell destruction in body, not loss of hemoglobin from body.

boiling the enzymatic digest with hydrochloric acid under a reflux. The enzymatic digests and the autolysates were boiled at pH 7.0 to destroy the enzymes and to remove the chloroform which was used as a preservative during incubation. They were then adjusted to pH 7.4, made isotonic, and diluted to a nitrogen content of 0.24 per cent. Immediately before use, they were again diluted with enough Tyrode solution to make the concentration of their nitrogen in the culture medium 0.06 to 0.003 per cent, according to the experiment.

Their effect was tested on fresh chicken blood monocytes, and also on pure strains of monocytes from blood and from spleen. The effect of increasing degrees of hydrolysis of the protein was tested by comparative experiments with the peptic, the tryptic, and the ereptic and tryptic digests of a given protein at equal nitrogen concentration. As a further test, experiments were made with the different digests in solutions containing the same amount of free amino nitrogen. As yet, no experiments have been undertaken with isolated or purified constituents of the digests.

The fresh blood monocytes were obtained from centrifuged blood. After removing the plasma, the leucocyte layer was coagulated with a drop of dilute tissue juice. The disk of leucocytes was removed, washed in Ringer solution, and cut into tiny squares. Adjacent sections of this leucocyte film were used for comparative experiments. The experimental media consisted of a mixture of equal parts of plasma and digest. The controls were cultivated in equal parts of plasma and Tyrode solution. When it was desired to test the digest combined with serum or Tyrode solution instead of plasma, only half as much plasma was used in the original clot and after coagulation, the cultures were washed twice at 39°C. with the respective experimental and control media. 1½ hours were allowed for each washing. On the 2nd day, the cultures were patched with a small quantity of the original medium and the washing was repeated. Fresh nutritive material was supplied every 2 or 3 days thereafter. This was done by incubating the cultures for 2 hours with the new fluid, after which the excess was drawn off. Diffusion between the old and the new medium was sufficient to wash away the waste products, neutralize the acid, and supply fresh nutritive material. Heparin 1:10,000 was used in the plasma to maintain its fluidity. This method of washing was adopted so as not to change the concentration of plasma and serum already contained in the coagulum.

The rate of cell multiplication was ascertained from the area of the colonies and the density of their cell population. The density was measured comparatively by examination of each culture under the microscope. A permanent record was obtained by photographing the colonies from many experiments. It is not possible by this means to measure small differences in the rates of multiplication, since the cells are ameboid and migrate over a large area. The technique is sufficiently accurate, however, for the purposes of these experiments. The differences observed between the control and experimental cultures in both area and density of cell population were so great as to prove beyond doubt that they have markedly

A-1383. Acute leukemia—hemoglobin 40 per cent—61 yrs.

Liver—autopsy weight 3500 gm.—laboratory specimen 3300 gm.

Histological specimen—advanced leukemic infiltration of liver with white cells.

Liver capillaries very rich in red cells. Liver cells show no pigment and a moderate grade of atrophy but in general are normal.

A-1231. Acute leukemia—hemoglobin 50 to 20 per cent—transfusions—terminal infection—13 yrs.

Liver—autopsy weight 1470 gm.—laboratory specimen 1420 gm.

Histological specimen—liver shows the usual leukemic infiltration in portal tissue and between the liver strands. There are small central hyaline liver necroses. Some liver cells show fat droplets. Kupffer cells show a yellow granular pigment which only occasionally gives a positive iron stain.

A-1956. Acute lymphatic leukemia—bleeding—transfusions—hemoglobin 67 per cent—9 yrs.

Liver—autopsy weight 1140 gm.—laboratory specimen 1100 gm.

Histological specimen—liver cells are normal. White cells are very numerous in all liver capillaries and there is conspicuous infiltration of the periportal stroma. Very little pigment is found anywhere. Kupffer cells are large. Occasional pigment granules within the liver cells give a positive stain for iron.

A-1688. Acute leukemia—hemoglobin 28 per cent—3 yrs.

Liver—autopsy weight 1020 gm.—laboratory specimen 920 gm.

Histological specimen—liver shows a typical white cell infiltration especially marked about the portal spaces. The liver cells show some fat droplets in the central portion of the lobules. Otherwise normal.

A-436. Acute leukemia—hemoglobin 60 per cent—42 yrs.

Liver—laboratory specimen 2300 gm.

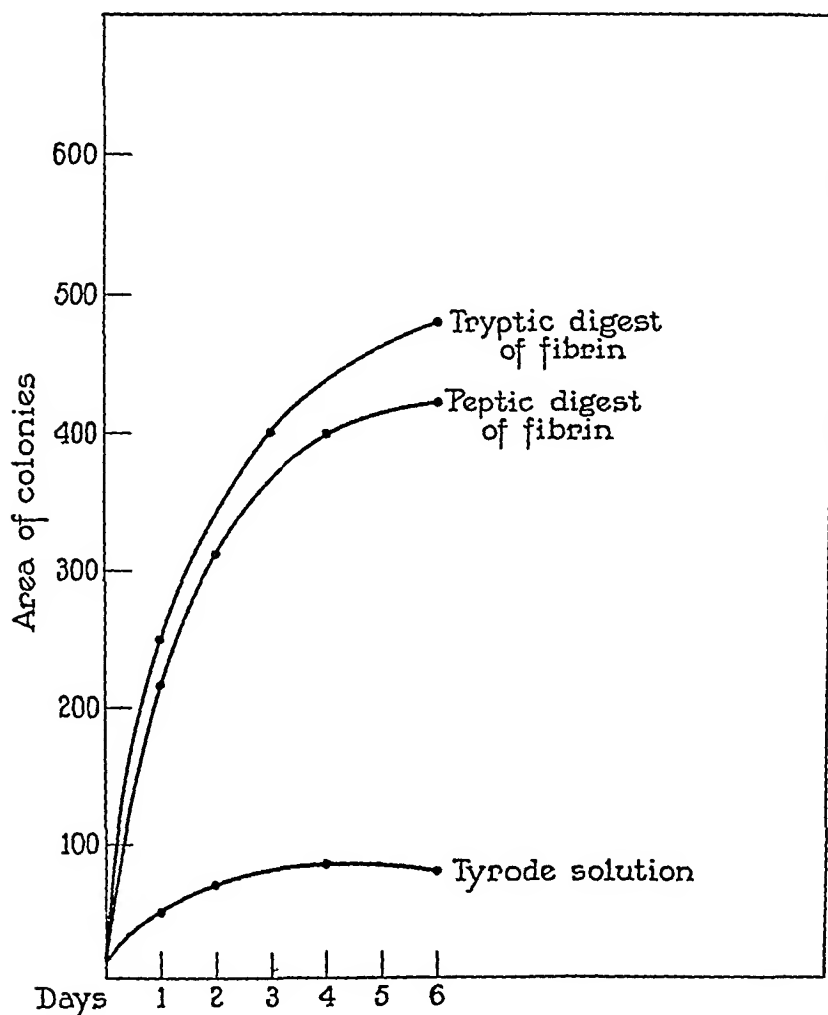
Histological specimen—premyelocytes fill capillaries. No eosinophiles. Central 1/2 of liver lobules shows much fatty degeneration. Outer 1/2 of lobule normal. Kupffer cells large and often show yellow granular pigment. Lipochrome scarce. Kupffer cells full of iron-containing pigment; hepatic cells contain no iron staining material.

A-385. Acute leukemia—hemoglobin 50 per cent—terminal infection—33 yrs.

Liver—laboratory specimen 2300 gm.

Histological specimen—central liver cells are much injured with fine fat droplets and poor staining. Some focal areas of necrosis in mid zone. Kupffer cells large and phagocytic. Many myeloblasts. Outer half of liver lobule well preserved and normal.

growth with increased dilution (Text-fig. 4). In others, the area of the colonies did not vary with the concentration. It is probable, therefore, that the rate of proliferation is limited by the amount of some other necessary constituent in the medium.



TEXT-FIG. 1. Experiment 4563 H. Comparison of area of colonies of fresh blood monocytes obtained in peptic digest of fibrin, tryptic digest of fibrin, and Tyrode solution. The coagulum was washed to remove the serum from the original clot.

It is not possible to explain fully at the present time the part played by serum or heparin plasma when combined with the digest. Serum and plasma are sufficient for maintenance of monocytes, but allow

cytes, most marked in the periportal tissue. The liver cells in general look normal but contain a few yellow pigment grains which stain for iron. Most of this pigment is in the margin of the lobule. Kupffer cells are large but contain no pigment.

A-77. Myelocytoma, subacute nephritis—hemoglobin 60 per cent—53 yrs.

Liver—laboratory specimen 1830 gm.

Histological specimen—few focal liver necroses, little central fat. Lipochrome moderate in amount, stroma normal. Congestion marked. No tumor in liver.

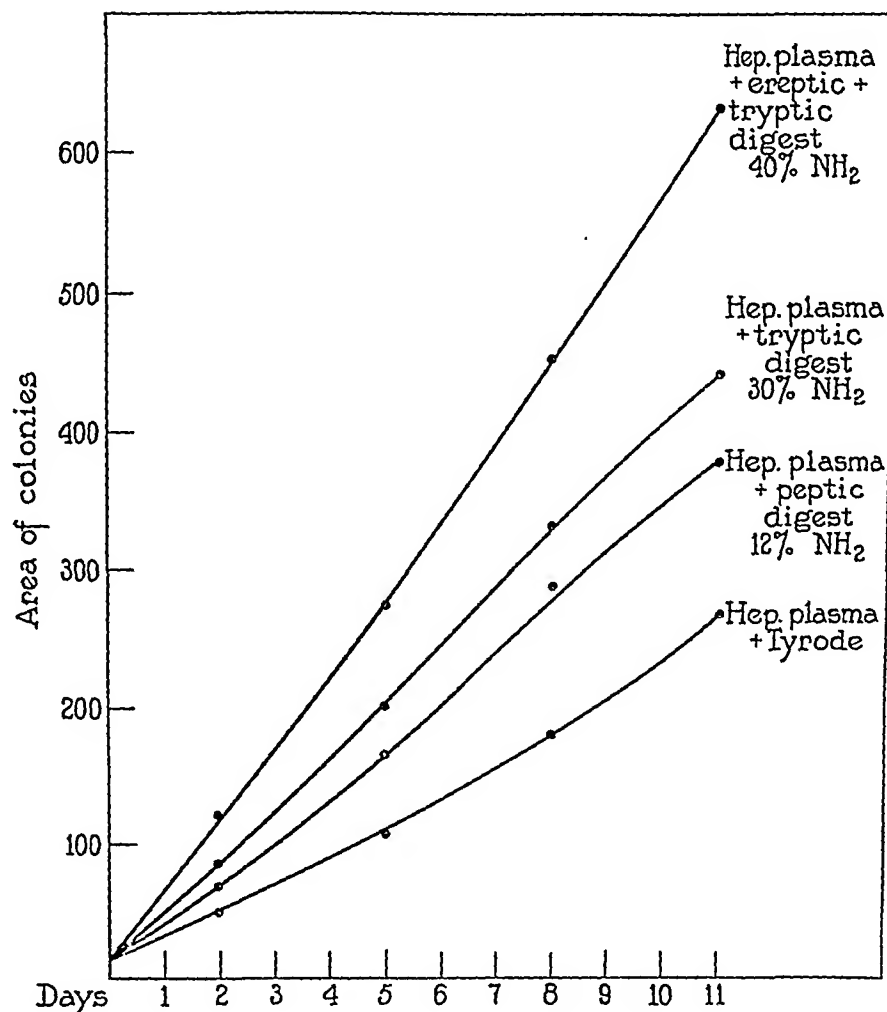
TABLE 34

Leukemias

Number	Cause of death	Iron content human liver		Liver intake per day		Hemoglobin output per 7 days feeding		
		fresh tissue	daily intake	Human	Control	from Human	from Control	ratio Human to Control
		mg. per cent	mg.	gm.	gm.	gm.	gm.	per cent
A-1769	Acute Leuk.	11.4	21	180	300	48	58	137
A-476	Acute Leuk.	24.5	59	225	300	57	54	139
A-1383	Acute Leuk.	13.5	62	470	300	118	56	134
A-1231	Acute Leuk.	10.9	22	200	200	61	62	98
A-1956	Acute Leuk.	9.4	14	157	300	45	60	87
A-1688	Acute Leuk.	8.6	11	130	300	53	72	171
A-436	Acute Leuk.	—	—	328	300	51	43	109
A-385	Acute Leuk.	—	—	329	300	47	34	127
A-377	Acute Leuk.	—	—	379	300	87	53	130
A-1963	Acute Leuk.	19.2	42	220	300	84	61	187
A-1349	Myeloid Leuk.	11.7	26	220	300	47	35	181
A-316	Myeloid Leuk.	—	—	283	225	46	30	121
A-1828	Lymphoid Leuk.	3.6	13	350	300	39	58	57
A-1943	Lymphoid Leuk.	16.4	74	450	300	101	81	84
A-77	Myeloma	—	—	261	300	50	43	135
Average.		12.9	34	279				126

Table 34 shows 15 cases of leukemia representing all types of the disease. There is one case of myeloma which perhaps does not belong in this group but at least the biological assay of liver potency falls close to the general average so that no harm is done. Biological assay of the hemoglobin producing factors shows a low normal figure (126 per cent) which corresponds to the assay of cases of secondary anemia (Table 33). The great majority of these cases are acute with large

monocytes, the presence of serum or plasma was as essential to the cultivation of the cells in tryptic and ereptic digests as to their cultivation in the digests consisting mostly of proteoses. It is evident,



TEXT-FIG. 3. Experiment 13261 D. Comparison of the areas of colonies of monocytes obtained in casein digests of equal concentration of nitrogen and varying degrees of hydrolysis. Heparin plasma was used with the digests.

therefore, that for these cells the serum plays some other part than that of a proteolytic enzyme. Its action may be due to a respiratory hormone or substances necessary to the fat or carbohydrate metabolism of the cells. The antitryptic activity of the serum is undoubtedly

extreme chloroform injury. No blood clots at autopsy indicate a serious liver insufficiency.

A-62. Eclampsia in last part of pregnancy (7 mos.). Child *in utero* at autopsy. 25 + yrs.

Liver—laboratory specimen 1725 gm.

Histological specimen—typical hemorrhagic periportal necroses of eclampsia.

Parenchyma elsewhere normal. Possibly 1/5 of liver cells or less are injured.

A-1261. Pulmonary embolism in normal puerperium—nursing—30 yrs.

Liver—autopsy weight 1700 gm.—laboratory specimen 1450 gm.

Histological specimen—liver in all respects normal.

TABLE 35

Miscellaneous

Number	Cause of death	Iron content human liver		Liver intake per day		Hemoglobin output per 7 days feeding		
		fresh tissue	daily intake	Human	Control	from Human	from Control	ratio Human to Control
		<i>mg. per cent</i>	<i>mg.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>
A-1367	Thalasemia	123.0	130	105	300	68	50	308
A-1819	Hemochromatosis	96.0	271	280	300	63	49	137
A-526	Marrow aplasia	68.0	208	300	142	52	38	65
A-1785	Chloroform	6.0	13	205	300	62	57	159
A-62	Eclampsia	—	—	150	400	0	39	0
A-1261	Embolism	5.9	12	200	142	18	38	33
	Lactation							

Table 35 contains cases of unusual interest which do not seem to fit in any of the tables given above. Each case deserves a special note.

Thalasemia (A-1367) is a term which we have used to designate a peculiar familial anemia found in races about the Mediterranean first described adequately by Cooley and recently from this laboratory (4). This disease presents some features resembling pernicious anemia and hemochromatosis. There are large deposits of iron-containing pigments in the liver, pancreas, viscera and ductless glands. It is not surprising that there should be a high potency for hemoglobin production in the liver assay—308 per cent. One should however contrast a typical case of *hemochromatosis* (A-1819) with more than twice the

did occur after a long time, when serum was used with the digests. This was not due to any difference in the antitryptic titre of plasma and serum. The antitryptic activity of serum and plasma taken from the same animal was tested by the Fuld-Gross^{5,6} method. It was found to be the same originally, and to decrease at the same rate over a period of 17 days.

Effect of the Digests on the Morphological Appearance of the Cells

The effect of these proteolytic digests on the morphological appearance of the cells was marked. Blood monocytes cultivated in plasma change gradually from small round forms to slender elongated cells, richer in mitochondria and neutral red granules than those that are starved in Tyrode solution.^{1,2} The addition of proteolytic digests to the plasma medium caused an increase in the size of the cell and an increase in the number of cytoplasmic structures or granulations over that which they acquire in plasma (Fig. 2 *a* and *b*). The cells became loaded with neutral red granules and contained somewhat more fat than those in normal medium. The very granular condition does not indicate that the monocytes are degenerating. They appear more like cells that are abundantly fed or even overfed. They multiply rapidly and continue to live for a long time. They migrate, and are shown by cinematography to have actively undulating membranes. The appearance of the cells changes continuously during their sojourn in these media. During rapid proliferation, chains and branching chains of cells are formed. After a considerable period of cultivation, they apparently anastomose and form groups of agglutinated cells over the entire medium (Fig. 3; see also Figs. 6 *d*, 8 *b*, 10 *b*, and 13). These agglutinated cells are still extraordinarily active. Cinematography shows that they are all in motion, pulling away from each other but apparently unable to pull themselves apart. Their membranes still undulate continuously. In some experiments, the whole medium between the cells was covered by these membranes, and every cell was joined to one or several other cells. In certain cultures, the interlocking of the monocytes was so great as to give the appearance of tissue formation.

⁵ Fuld, E., *Arch. exp. Path. u. Pharmacol.*, 1908, 58, 468.

⁶ Gross, O., *Arch. exp. Path. u. Pharmacol.*, 1907, 57, 157

another different but related substance under different demand or emergency conditions. We hope to obtain further information about lactation and the storage of these potent hemoglobin producing factors in the liver.

SUMMARY

Biological assay of the human liver in various types of anemia shows conspicuous differences in the concentration of hemoglobin producing factors.

Pernicious anemia shows very high values and the liver in untreated cases may show maximal storage of the hemoglobin producing factors. Liver therapy reduces this store as the missing factor is supplied and new hemoglobin and red cells can be turned out by the marrow.

Aplastic anemia likewise shows high concentration of hemoglobin producing factors as there is no outlet for this material through the red marrow.

Secondary anemia due to loss of blood will show low normal values but even long standing severe anemia will not seriously deplete this store of hemoglobin producing factors in the liver.

Secondary anemia due to blood destruction within the body shows higher values and some excess store of hemoglobin producing factors and iron.

Leukemia gives a biological assay like secondary anemia due to blood loss and always presents definite anemia.

Iron analyses show conspicuous differences and iron concentration within the liver parenchyma does not in any way parallel the concentration of hemoglobin producing factors. The highest values for iron concentration are found in aplastic anemia (70 mg. per cent)—high values in pernicious anemia (51 mg. per cent)—normal values in leukemia (13 mg. per cent)—and low values in anemia due to loss of blood (5.3 mg. per cent).

These findings should aid in a more complete understanding of the pathogenesis and internal metabolism of various anemias.

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2. Richter, O., Ivy, A. C., and Kim, M. S., *Proc. Sec. Exp. Biol. and Med.*, 1932, 29, 1093.
3. Whipple, G. H., *Arch. Int. Med.*, 1922, 29, 711.
4. Whipple, G. H., and Bradford, W. L., *Am. J. Dis. Child.*, 1932, 44, 336.

first in the ereptic and tryptic digest (Fig. 6 *d*), next in the tryptic digest, and much later, if at all, in the peptic digest.

The acid-hydrolyzed proteins and the mixture of pure amino acids had no effect on the morphology of the cells comparable to that of the enzymatic digests. The amino acid mixtures, if dilute, caused no noticeable change in the cells. When used at higher concentrations, the cells became round and vacuolated, and degenerated. In the protein completely hydrolyzed by acid, the cells became somewhat broader and more granular than the control cells, but did not undergo the marked changes produced by enzyme-hydrolyzed material. These changes cannot be ascribed, therefore, to the amino acids in the digests. They may be due to the intermediate products of protein hydrolysis. All the digests used in these experiments, even those having the largest quantity of amino nitrogen, were found to undergo further hydrolysis on treatment with acid. There is also the possibility that the digests contain small quantities of some material that supplements the amino acids or the peptides.

The concentration of the digest in the medium plays as important a part as its degree of hydrolysis in determining the size and shape of the cells and their tendency to agglutinate. As the concentration is increased, the cells become shorter, broader, and more granular; (compare Fig. 7 *b* with 7 *c*, also Fig. 9 *a* with 10 *a*). Because of this, cells cultivated in a high concentration of tryptic digest (30 per cent amino nitrogen) may resemble those cultivated in a lower concentration of peptic digest (12 per cent amino nitrogen) (compare Fig. 7 *c* with 7 *a*). The higher the concentration of the digests, the more quickly agglutination of the cells takes place. At 0.01 per cent nitrogen, agglutination in a tryptic digest of fibrin took place in 24 days. At 0.03 per cent, the same effect was observed in 15 days. In the less hydrolyzed peptic digest, no agglutination was observed at 0.01 per cent nitrogen during the entire period of cultivation. It did take place, however, in a more concentrated solution.

The length of time of cultivation in these media is as important in determining the morphology of the cells as the degree of hydrolysis or the concentration of the digest. The increase in size of the cell and in the number of its cytoplasmic structures is a gradual process. Also there is a cumulative effect of the digest as the time of cultivation is

THE EFFECT OF PROTEOLYTIC DIGESTION PRODUCTS ON MULTIPLICATION AND MORPHOLOGICAL APPEARANCE OF MONOCYTES

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PLATES 42 TO 44

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It is known that blood monocytes and tissue macrophages will subsist upon a medium of serum or plasma diluted with Tyrode solution.^{1,2} They also feed upon bacterial protein, muscle tissue, and coagulated protein such as egg albumin.^{1,2} Embryonic juice and proteoses, if sufficiently dilute, cause active multiplication of these cells, but kill them under conditions that promote the multiplication of fibroblasts.¹⁻³ It is also known that the morphological characteristics of the monocytes vary according to their nutritive state.^{1,2} Although it seems evident that the monocytes live on digested protein, no detailed study has heretofore been made of the effect of various products of protein digestion on these cells. The experiments reported in this paper deal with the effect of proteins hydrolyzed to varying degrees on (1) the rate of multiplication, and (2) the morphological appearance of monocytes cultivated *in vitro*.

Technique

Commercial blood fibrin, Harris purified casein, and freshly extirpated chicken liver were used as the source of the protein hydrolytic products. By digesting with pepsin, trypsin, and a combination of trypsin and erepsin, digests were obtained in which the amount of nitrogen in the amino form varied from 12 to 68 per cent of the total nitrogen. Proteolytic products were also obtained by the autolysis of fresh liver tissue at pH 4.5. Completely hydrolyzed protein was prepared by

¹ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1926, 44, 285.

² Carrel, A., *Science*, 1931, 73, 297.

³ Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1928, 47, 553, 571; 48, 533. Baker, L. E., *J. Exp. Med.*, 1929, 49, 163.

1:20,000 to 1:80,000, agglutinated masses of the leucocytes are formed of the same character as those produced by hydrolyzed proteins (Figs. 13 and 14). The monocytes cultivated in the presence of arsenic pentoxide acquire the property of liquefying the plasma clot. Too little evidence is at hand as yet to indicate whether enough digestion products are formed in the liquefaction to cause this change, or whether the arsenic oxide brings about the agglutinations by an entirely different mechanism. The arsenic pentoxide does not cause any such marked increase in monocyte proliferation as is obtained in the proteolytic digests.

If the monocytes are cultivated for too long a time in these media, they finally degenerate. For continued life, they must be transferred occasionally to their ordinary medium of plasma and Tyrode solution. When this is done, they gradually revert to the condition of cells that have been cultivated in normal medium. This reversible change is, however, very slow. The cells sometimes retain the morphological appearance that they had in the medium containing proteolytic products for as long as 14 days after they have been restored to normal medium. The slow return of the cell to its normal condition suggests that it may be necessary in considering cell morphology to take into account the past history of the cell, as well as its present environment. Quite often giant cells are formed (Fig. 12). Although their occurrence is not specific to cultivation in proteolytic digests, it shows still another kind of cell reaction that has been observed in these media.

SUMMARY

It has been found that the enzymatic digestion products of proteins cause a rapid proliferation of blood monocytes *in vitro*. Digestion mixtures having anywhere from 12 to 68 per cent of their nitrogen in the amino form possess this property.

For continued proliferation, heparin plasma or serum must be repeatedly supplied with the digests. Without them, multiplication continues for only a few days, the coagulum in which the cells are embedded liquefies around the central fragment, and the cells disintegrate.

The enzymatic digests exert a marked effect on the morphological appearance of the cells and finally cause them to agglutinate. The extent of this effect is determined by the degree of hydrolysis of the

different rates of cell proliferation. Measurements could be made only during the 1st week or 10 days of cultivation. After that time, small colonies of cells developed over the entire surface of the coagulum.

RESULTS

Effect of Proteolytic Products on the Multiplication of Monocytes

The addition of any of the enzymatic digests to the culture medium caused a large increase in the rate of proliferation of monocytes in leucocytic film. The same result was obtained in pure strains of monocytes from blood and from spleen. When the serum was washed out of the clot in which the leucocytes were embedded and only the proteolytic products were supplied as nutritive material, proliferation continued for a few days at a rate greatly exceeding that in Tyrode solution (Text-fig. 1), but the cells survived only 7 or 8 days. Digestion of the coagulum also occurred around the central fragment. When, however, either serum or heparin plasma was provided in addition to the digests, rapid proliferation continued for a much longer time, and the cells survived for many weeks without deterioration. All the results described below are taken, therefore, from experiments in which either serum or heparin plasma was used with the proteolytic products. The increased proliferation due to the digests was evident both from the larger area of the colonies obtained (Text-figs. 2 and 3) and from the greater density of the cell population in that area (Fig. 1 *a* and *b*). The same phenomenon is evident in Figs. 5 and 6.

The digests that contained small amounts of free amino nitrogen did not cause as rapid cell multiplication as the ones containing larger amounts of the lower split products (Text-figs. 2 and 3). The autolysates of liver also promoted cell proliferation. No comparable effects could be obtained with protein completely hydrolyzed by hydrochloric acid or with artificial mixtures of pure amino acids. The acid-hydrolyzed protein stimulated the multiplication very slightly. Not even the smallest increase in proliferation could be observed on adding mixtures of pure amino acids to the medium.

High concentrations of the digests were toxic to monocytes. At the concentrations used (0.024 per cent nitrogen to 0.006 per cent nitrogen), some of them, especially the liver digests, gave larger

EXPLANATION OF PLATES

Magnification = $\times 230$ in all the figures.

PLATE 42

FIG. 1. Experiment 13352. Effect of proteolytic digest on density of cell population.

Fig. 1 *a*. Control. Fresh blood monocytes cultivated for 3 days in heparin plasma and Tyrode solution.

Fig. 1 *b*. Fresh blood monocytes cultivated for 3 days in heparin plasma and an ereptic and tryptic digest of fibrin (nitrogen = 0.024 per cent; amino nitrogen = 40 per cent of the total).

FIG. 2. Experiment 13342. Effect of slightly hydrolyzed protein on the morphology of the cell.

Fig. 2 *a*. Fresh blood monocytes cultivated 16 days in heparin plasma and Tyrode solution.

Fig. 2 *b*. Fresh blood monocytes cultivated 16 days in heparin plasma and peptic digest of fibrin (amino nitrogen = 15 per cent of the total).

FIG. 3. Experiment 3865 H3. Agglutinations of monocytes cultivated 19 days in heparin plasma and tryptic digest of liver (nitrogen = 0.008 per cent; amino nitrogen = 56 per cent of the total). Note the pseudopod-like folds in their undulating membranes.

FIG. 4. Experiment 3987 H4. Effect of more highly digested protein on the morphology of the cell. Monocytes kept for two passages (24 days) in heparin plasma and tryptic and ereptic digest of fibrin (nitrogen = 0.006 per cent; amino nitrogen = 43 per cent of the total). The control cells in heparin plasma and Tyrode solution not pictured here were indistinguishable from those shown in Fig. 2 *a*.

FIG. 5. Experiment 12944 D. Lengthening of cells in pure strain of macrophages, due to tryptic digest of chicken liver.

Fig. 5 *a*. Control cultivated 3 days in serum and Tyrode solution.

Fig. 5 *b*. Same monocytes cultivated 3 days in serum and tryptic digest of chicken liver (amino nitrogen = 56 per cent of the total).

PLATE 43

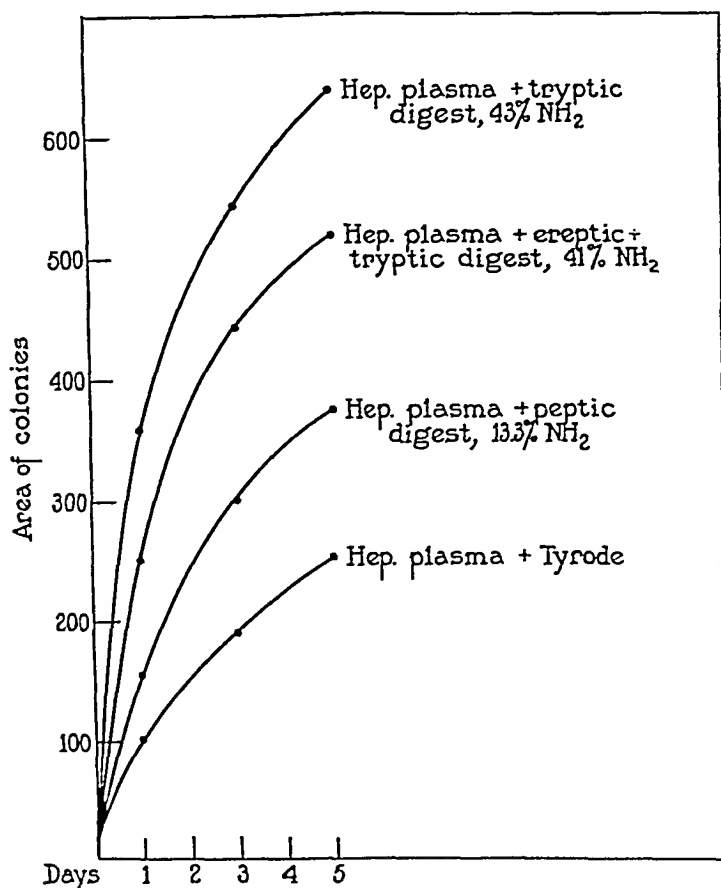
FIG. 6. Experiment 13261 D. Effect of increasing degrees of hydrolysis of casein.

Fig. 6 *a*. Control cultivated 13 days in heparin plasma and Tyrode solution.

Fig. 6 *b*. Fresh blood monocytes cultivated 13 days in heparin plasma and peptic digest of casein (nitrogen = 0.024 per cent; amino nitrogen = 12 per cent of total nitrogen). Note increase in the number of cells.

Fig. 6 *c*. Fresh blood monocytes cultivated 13 days in heparin plasma and tryptic digest of casein (nitrogen = 0.024 per cent; amino nitrogen = 30 per cent

only a slow proliferation. The proteolytic products in Tyrode solution cause an initial rapid proliferation, but are not in themselves adequate for maintenance. Willmer and Kendal⁴ have reported that



TEXT-FIG. 2. Experiment 4009 H. Comparison of the areas of colonies of monocytes obtained in heparin plasma and fibrin digests of varying degrees of hydrolysis with those obtained in heparin plasma and Tyrode solution. The digests were used at equal concentrations of nitrogen.

serum is necessary for the cultivation of fibroblasts in proteose solutions, and attribute the action of the serum to an enzyme that breaks down the proteose to lower disintegration products. In the case of

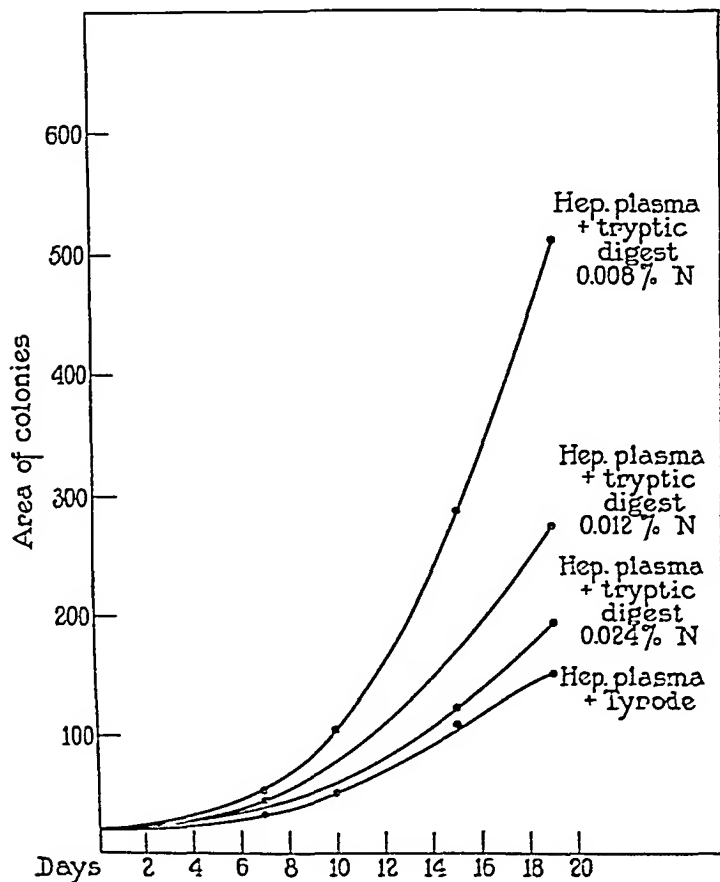
⁴ Willmer, E. N., and Kendal, L. P., *J. Exp. Biol.*, 1932, 2, 149.

FIG. 13. Experiment 13158 D. Agglutinations in monocytes cultivated in heparin plasma and tryptic digest of liver (nitrogen = 0.012 per cent; amino nitrogen = 70 per cent of the total nitrogen).

FIG. 14. Experiment 13043 D. Agglutinations in monocytes cultivated in heparin plasma and arsenic pentoxide 1:20,000.

important since without it the proteolytic digests cause liquefaction of the coagulum.

Both serum and heparin plasma supplied the substances necessary for survival and rapid proliferation of monocytes in the hydrolyzed



TEXT-FIG. 4. Comparison of the areas of monocytes obtained in a tryptic digest of liver at different concentrations. Heparin plasma was used with the digests.

proteins. However, the cells became more granular when serum was used, and underwent changes in morphology more rapidly than when heparin plasma supplemented the digests. In the presence of heparin plasma, no liquefaction of the coagulum was observed. Some digestion

FIG. 13. Experiment 13158 D. Agglutinations in monocytes cultivated in heparin plasma and tryptic digest of liver (nitrogen = 0.012 per cent; amino nitrogen = 70 per cent of the total nitrogen).

FIG. 14. Experiment 13043 D. Agglutinations in monocytes cultivated in heparin plasma and arsenic pentoxide 1:20,000.

The change in morphology that the cells undergo in these media is determined by (1) the degree of hydrolysis of the digest, (2) its concentration in the medium, and (3) the length of time the cells are cultivated in the digests. Cells cultivated in serum and digest also differ morphologically from those cultivated in heparin plasma and digest. Those at the center of the colony differ from those at the periphery. Individual characteristics of the plasmas used with the digests also play a part in determining their exact morphological characteristics.

The degree of hydrolysis of the digests used in the medium determines the size, shape, degree of granulation of the monocytes, and their tendency to agglutinate. Monocytes cultivated in protein digests containing small amounts of amino nitrogen become larger, rounder, and more granular than those cultivated in plasma (Fig. 2 *a* and *b*). In the more highly hydrolyzed digests, the cells maintain the elongated form characteristic of those in plasma, but are much larger and longer than those in plasma (Fig. 5 *a* and *b*). In the second passage, these differences are very marked (Fig. 4). The control cells not pictured here were indistinguishable from the controls pictured in Fig. 2 *a*.

The effect of increasing degrees of hydrolysis of a given protein is illustrated in Fig. 6 *a*, *b*, *c*, and *d*. The same plasma was used with Tyrode solution (*a*), peptic digest of casein (*b*), tryptic digest of casein (*c*), and ereptic and tryptic digest of casein (*d*), at an equal concentration of total nitrogen. The cells in the ereptic and tryptic digest are somewhat shorter and broader than those in the tryptic digest, and show the beginning of agglutination. Similar differences in the cells in peptic and tryptic digests of fibrin are also shown in Fig. 7 *a* and *b*. When the digests were used at such dilution that their concentrations of free amino nitrogen were the same, there was less difference between the cells of the ereptic and the tryptic digests than at equal total nitrogen. The cells of the peptic digests still differed in character from the others because of the presence of the larger fractions of the protein molecule.

The time required for the cells to agglutinate in these media depends also on the degree of hydrolysis of the digest. When the digests are used at equal concentrations of total nitrogen, the cells agglutinate

FIG. 13. Experiment 13158 D. Agglutinations in monocytes cultivated in heparin plasma and tryptic digest of liver (nitrogen = 0.012 per cent; amino nitrogen = 70 per cent of the total nitrogen).

FIG. 14. Experiment 13043 D. Agglutinations in monocytes cultivated in heparin plasma and arsenic pentoxide 1:20,000.

extended. Within certain limits, the same results can be produced by long cultivation at low concentration as occur at a higher concentration in a short time. It is also possible for two digests of different degrees of hydrolysis to produce the same result, but the length of time required to give this result is different.

The location of a cell in the colony also plays a part in determining its morphology. Those at the periphery of the colony were broader and rounder than those near the central fragment. They were also more granular, contained more fat, and showed anastomoses first (Fig. 8 *a* and *b*). This is to be expected since the concentration of the chemical constituents of the medium is altered by the metabolism of the cells. The pH and the concentration of the nutritive substances are changed more rapidly at the center than at the periphery of the colony.

The cells cultivated in serum and a given digest differed in morphology from those cultivated in plasma and the same digest (Figs. 9 and 10). In serum and digest, the cells were larger than in heparin plasma and digest and appeared much more granular. Agglutinations also occurred more quickly when serum supplemented the digest than when heparin plasma was used with it (Fig. 10 *a* and *b*). These differences were observed even when the serum and plasma were taken from the same animal.

The character of the plasma used with the digest also plays some part in determining the morphology of the cell. Plasmas taken from different animals of the same species vary over a considerable range in the quantity of some of their constituents. Six different plasmas, when combined with the same digest of casein at a given concentration, produced cells of varying lengths. Fig. 11 *a* and *b* shows two extremes in this experiment. However, the part played by the plasma is quite secondary to that of the digests. In order to obtain the types of cells and growth pictured in this paper, it is necessary for the medium to contain proteolytic products.

The agglutination of the leucocytes into masses of cells is a very striking phenomenon. It has so far been observed under only one other condition of cultivation; namely, by the addition of a very dilute solution of arsenic pentoxide to the usual culture medium of plasma and Tyrode solution. In concentrations of arsenic pentoxide from



digest, its concentration, and the length of time the cells are cultivated in it. The morphological appearance of the cells is also somewhat influenced by the nature of the plasma or serum used with the digest.

Digests having very little free amino nitrogen produce short, round, granular cells. Those more highly hydrolyzed produce large, long, slender forms.

An increase in the concentration of the digest in the medium causes a shortening and broadening of the cell and an increase in its granulations. Therefore, even a highly hydrolyzed digest may, if concentrated, give cells resembling those in a lower concentration of a less hydrolyzed one.

The digests have a cumulative effect on the cells, as the time of cultivation is extended. Therefore, cultivation for a long period in a dilute solution may give the same effect as a shorter time in a higher concentration.

A different effect is obtained if plasma is used with the digest than if serum is used, even when the plasma and serum are taken from the same animal. The monocytes cultivated in serum and digest are generally shorter, broader, and more granular than those cultivated in heparin plasma and digest. They also contain more fat and have a greater tendency to digest the clot.

Agglutination of the cells takes place more readily in highly hydrolyzed products than in those slightly hydrolyzed. It is hastened by increase in concentration of the digest in the medium. It occurs more readily at the periphery of the culture and sooner in serum and digest than in heparin plasma and digest.

Completely hydrolyzed proteins and mixtures of pure amino acids do not produce effects at all comparable to those of the enzymatic digests either in their effect on the rate of cell proliferation or their action on the morphology of the cell.

Arsenic pentoxide in dilutions from 1:20,000 to 1:80,000 is the only other agent known to bring about agglutinations of the monocytes when cultivated *in vitro*.

The early changes in the morphological appearance of the cell that are produced by these digests are reversible. When the digests are removed from the medium and the cells cultivated in plasma and Tyrode solution, they very gradually revert to their original form.



of the total nitrogen). Note the increased size and length of the cells over those in Fig. 6 *a* and *b*.

Fig. 6 *d*. Fresh blood monocytes cultivated 13 days in heparin plasma and tryptic and ereptic digest of casein (nitrogen = 0.024 per cent; amino nitrogen = 40 per cent of the total nitrogen). Note the beginning of agglutinations.

FIG. 7. Experiment 13343 D. Relative effects of concentration and degree of hydrolysis of digests on the morphology of the cell.

Fig. 7 *a*. Monocytes cultivated 14 days in heparin plasma and a peptic digest of casein (nitrogen = 0.01 per cent).

Fig. 7 *b*. Monocytes cultivated 14 days in heparin plasma and a tryptic digest of casein (nitrogen = 0.01 per cent).

Fig. 7 *c*. Monocytes cultivated 14 days in heparin plasma and a tryptic digest of casein three times as concentrated as in Fig. 7 *b*. Note the difference in the cells of Fig. 7 *a* and *b*, and the similarity of the cells in Fig. 7 *a* and *c*.

FIG. 8. Experiment 3987 H2.

Fig. 8 *a*. Cells near the central fragment in 15 day old culture cultivated in heparin plasma and peptic digest of fibrin.

Fig. 8 *b*. Cells at the periphery of the same culture after 15 days' cultivation.

PLATE 44

FIG. 9. Experiment 4573 H. Differences between the effects of heparin plasma and serum when combined with a tryptic digest of fibrin.

Fig. 9 *a*. Monocytes cultivated 15 days in heparin plasma and tryptic digest of fibrin (nitrogen = 0.024 per cent).

Fig. 9 *b*. Monocytes cultivated 15 days in serum and the same tryptic digest of fibrin (nitrogen = 0.024 per cent). Note the agglutinations of the round granular cells.

FIG. 10. Experiment 14161 D. Differences between the effects of heparin plasma and serum when combined with a tryptic digest of fibrin.

Fig. 10 *a*. Monocytes cultivated 19 days in heparin plasma and tryptic digest of fibrin (nitrogen = 0.06 per cent). These cells are round instead of long, as in Fig. 9 *a*, because of the greater concentration of the digest used in this experiment.

Fig. 10 *b*. Monocytes cultivated 19 days in serum and tryptic digest of fibrin (nitrogen = 0.06 per cent). Note the difference in the agglutinating effect of the serum, and also, by comparing Fig. 10 *b* with Fig. 10 *a*, the effect of increased concentration on agglutination.

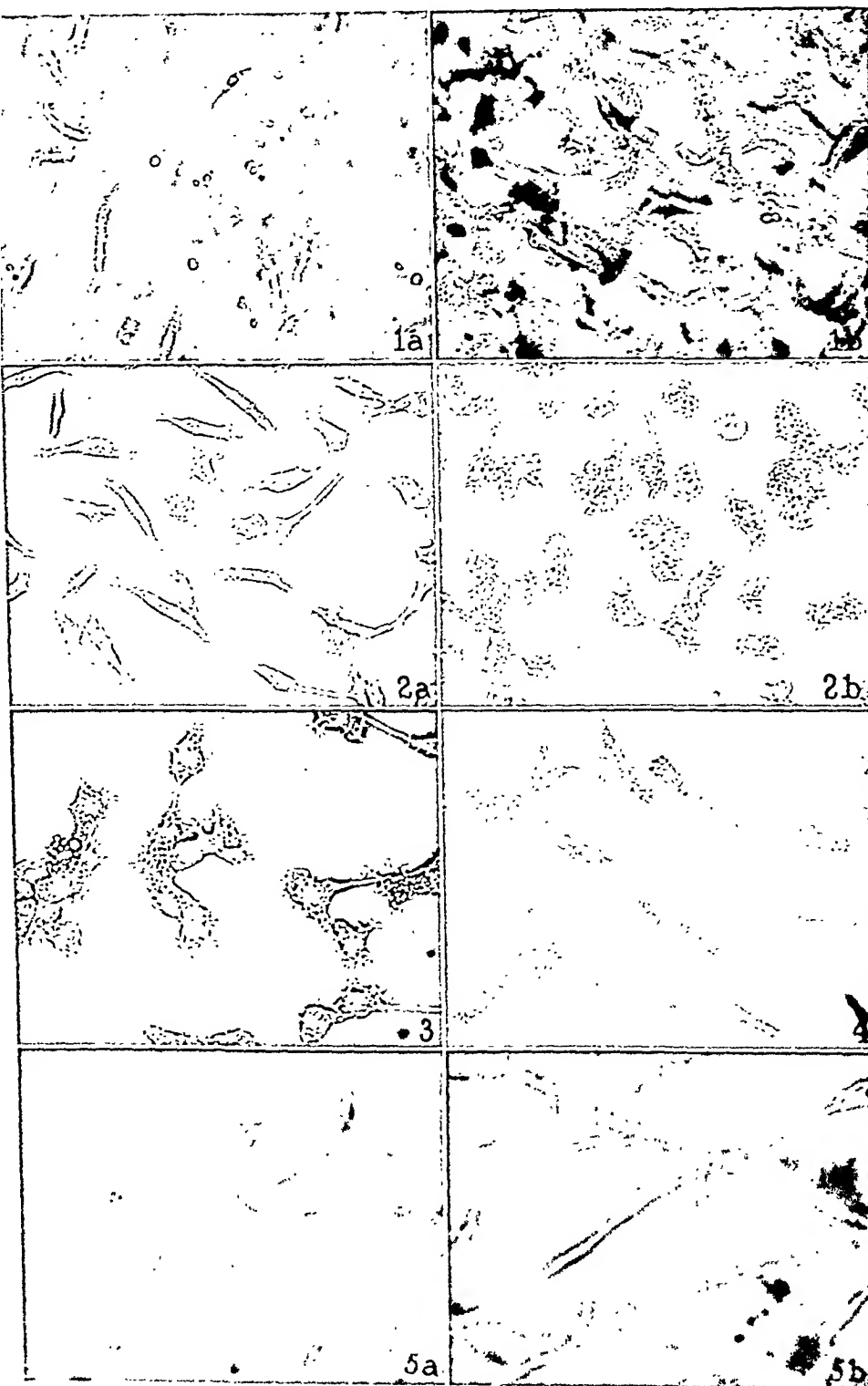
FIG. 11. Experiment 14270 D. Effect of two different plasmas with the same proteolytic digest.

Fig. 11 *a*. Monocytes cultivated in Plasma 1 with tryptic digest of casein (nitrogen = 0.024 per cent).

Fig. 11 *b*. Monocytes cultivated in Plasma 2 with the same tryptic digest of casein (nitrogen = 0.024 per cent).

FIG. 12. Experiment 3715 H. Giant cell resulting from cultivation of monocytes in tryptic digest of casein and later cultivation in serum. Note the undulating membrane at the end of the giant cell and surrounding the smaller cells.





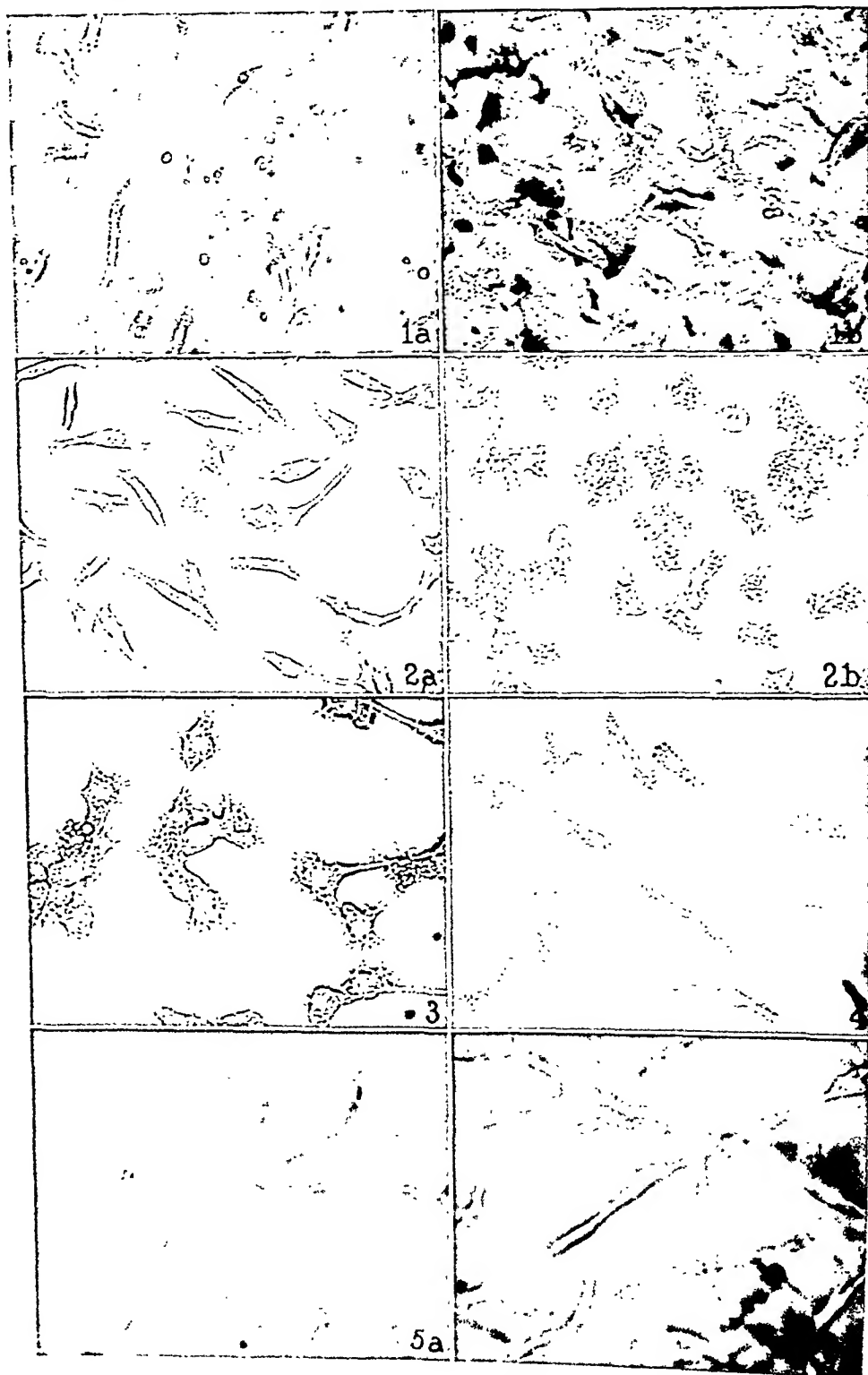
The subject is reviewed by Weech and Ling (4) who take up the story with the so called war edemas which occurred so frequently in the Central Empires when large groups of people were forced to subsist on inadequate diets low in protein and high in certain types of vegetables. They proceed to discuss cases of nutritional edema (with hypoproteinemia) in the Chinese from the famine districts and they point out that the patients had been subsisting on a diet composed chiefly of wheat, rice, corn, and millet, inadequate in protein as well as in vitamins and salts. Among the many recent papers emphasizing the same point of view may be mentioned those of Peters (5) and his associates. Peters (3) also feels that malnutrition as well as protein loss is an important factor in lowering the blood proteins in Bright's disease.

It should be made clear that those who adhere strictly to the protein lack theory of malnutritional hypoproteinemia attribute the disturbance of the blood proteins specifically to a shortage of protein building stones and not to an interference with the protein-building mechanism. Peters, for example, in commenting on rises of blood protein in malnourished patients after they were placed on a high protein diet says "the gradual rise of the serum proteins which accompanied the protein storage in these cases suggests that the low serum proteins resulted from protein deficiency alone" (5).

3. *Experimental Hypoproteinemia in Animals on Low Protein Diets.*—Kohman (6) several years ago found that rats placed on a low protein diet with large amounts of carrots became edematous after a number of weeks. While no blood protein determinations were made it seems certain that the edema was associated with hypoproteinemia. In analyzing her experiments Kohman felt that vitamin deficiency, salt effects, and possible toxic substances in carrots were all eliminated as causal factors and that the edema was due purely to lack of protein in the diet. Frisch, Mendel, and Peters (7) conducted similar experiments but on a less extensive scale. They confirmed Kohman's observations and made determinations of blood proteins which were found after a period of weeks to be very low. Shelburne and Egloff (8) report observations on a dog in which the blood proteins fell during a period of 24 days of low protein feeding from 6.8 gm. per cent to 4.7 gm. per cent.

4. *Experimental Hypoproteinemia Produced by Withdrawal of Blood Proteins (Plasmapheresis).*—Whipple and his associates (9) in connection with studies on the regeneration of blood proteins produced marked hypoproteinemias in dogs by removing large amounts of plasma (several hundred cubic centimeters). Leiter (10) and Barker and Kirk (11) in efforts to produce experimental edema were able to lower the blood proteins of dogs by similar drastic bleedings of 400 to 500 cc. twice daily. Fishberg and Fishberg (12) reduced the plasma proteins in rabbits to about 50 per cent of the normal value by daily bleedings of about 35 cc.

Certainly the array of evidence presented above can leave no doubt in any mind that loss of protein and lack of protein play an important

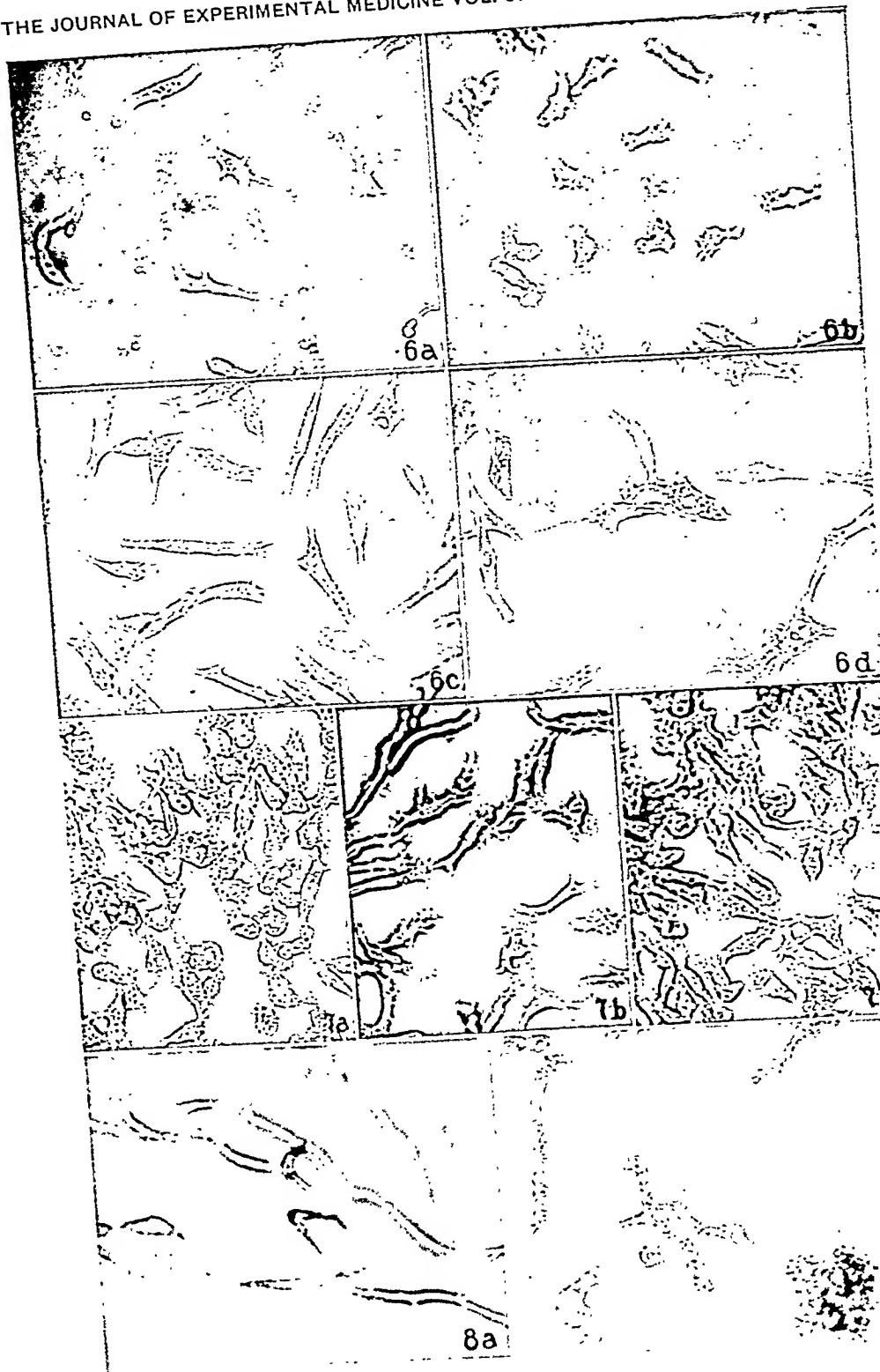


(Baker. Effect of digestion products on monocytes)

slightly depressed in dogs which went without food for 4 days; the globulins were found to be increased at the expense of the albumins. Then there are so many irreconcilable contradictions. Shelburne and Egloff's dog (8), for example, suffered a marked lowering of blood proteins within 3 weeks when placed on a protein diet, whereas Whipple's dogs (9), in spite of an initial large bleeding followed by total fasting, regenerated their plasma proteins to normal within 14 days. The whole subject evidently requires more experiment before final conclusions can be drawn.

We may next return to a consideration of the relation of proteinuria to the blood proteins of Bright's disease. If this loss of protein in the urine were the major factor one would expect the blood proteins to bear some relation to the degree of such loss and one would also expect the blood proteins to return to a higher level if large quantities of protein were fed. With regard to the first point, Fig. 3 from the paper by Peters, Bruckman, Eisenman, Hald, and Wakeman (3) is of interest. The urine protein in grams per day is plotted against the serum protein per cent, and it appears that there is no exact relation. Some patients with very heavy proteinurias (over 10 gm. per day) had plasma proteins of a moderately low (4.5 gm. per cent or over) whereas a good many subjects with urine proteins under 10 gm. had serum proteins under 4 gm. per cent. The extremes were urine protein 1 gm. per day with serum proteins 3+ gm. per cent, and urine proteins 19 gm. per day with serum proteins 4.5 gm. per cent. Furthermore, patients with the most marked proteinurias do not continue to lower the blood proteins indefinitely but they seem to establish them at a more or less constant low level despite the continued loss.

Even more striking are certain observations, such as those of Peters and Bullock (16), on the effect of high protein feeding on the low blood proteins in nephritis. Regardless of the diet and regardless of whether the patient was storing or losing nitrogen the blood proteins in a number of cases remained at a practically constant level. Their Case 2 is a good illustration. During the first period of 17 days the patient had a positive balance of 0.7 gm. protein per day but lost 10.8 gm. protein per day in the urine. Despite this loss the plasma proteins at the start were 4.03 gm. per cent and on the 12th day 4.20 gm. per cent. During the following 26 day period there was a positive daily balance of 22.7 gm. of protein, whereas 9.3 gm. were lost each day in the urine. The plasma proteins at the beginning of the period were 4.20, at the end 4.34. The patient's weight is not stated but if one estimates his total plasma as 3000 cc. then he had a total of 126 gm. of plasma protein at the start and 130 gm. at the end of the period. Although he had stored 348 gm. of protein only 4 gm. were diverted to the formation of plasma protein. These considerations make it clear that, in this case at least, the low blood proteins bore no relation to the nitrogen metabolism of the body as a whole but clearly were dependent on some special failure of the blood protein-forming mechanism which may have been exaggerated by the loss of protein in the urine. This special instance is in accord with general clinical experience and while various writers



regenerating mechanism; lack and loss undoubtedly contribute an added burden which, on occasion may be insuperable, but lack and loss clearly fail to explain the whole problem.

It would serve no purpose to hypothecate an indefinite defect of the protein-forming mechanism without proposing some explanation for such a defect. In the clinical cases, apart from protein lack in the diet, deficiencies of the diet in other respects, infection, intoxication from wasting disorders such as cancer, and the hardships and hazards of famine conditions have been outstanding, and they have been stressed by various writers. Any or all of these factors might be imagined to lead (apart from lack of protein *per se*) to an interference with the blood protein-forming mechanism, which perhaps readjusts itself when infection is eliminated and the general nutrition is improved. One must also consider the possibility of some more specific agent which may have a positive inhibiting effect on the blood protein-forming mechanism. In many of the famine cases as well as in experiments the diet has not only been low in protein but there has been an excess of certain vegetables such as turnips (war edema, Shelburne and Egloff's dog), and carrots (Kohman's and Frisch's rats). May these vegetables and perhaps other articles of food contain some agent which is antagonistic to the formation of blood proteins?

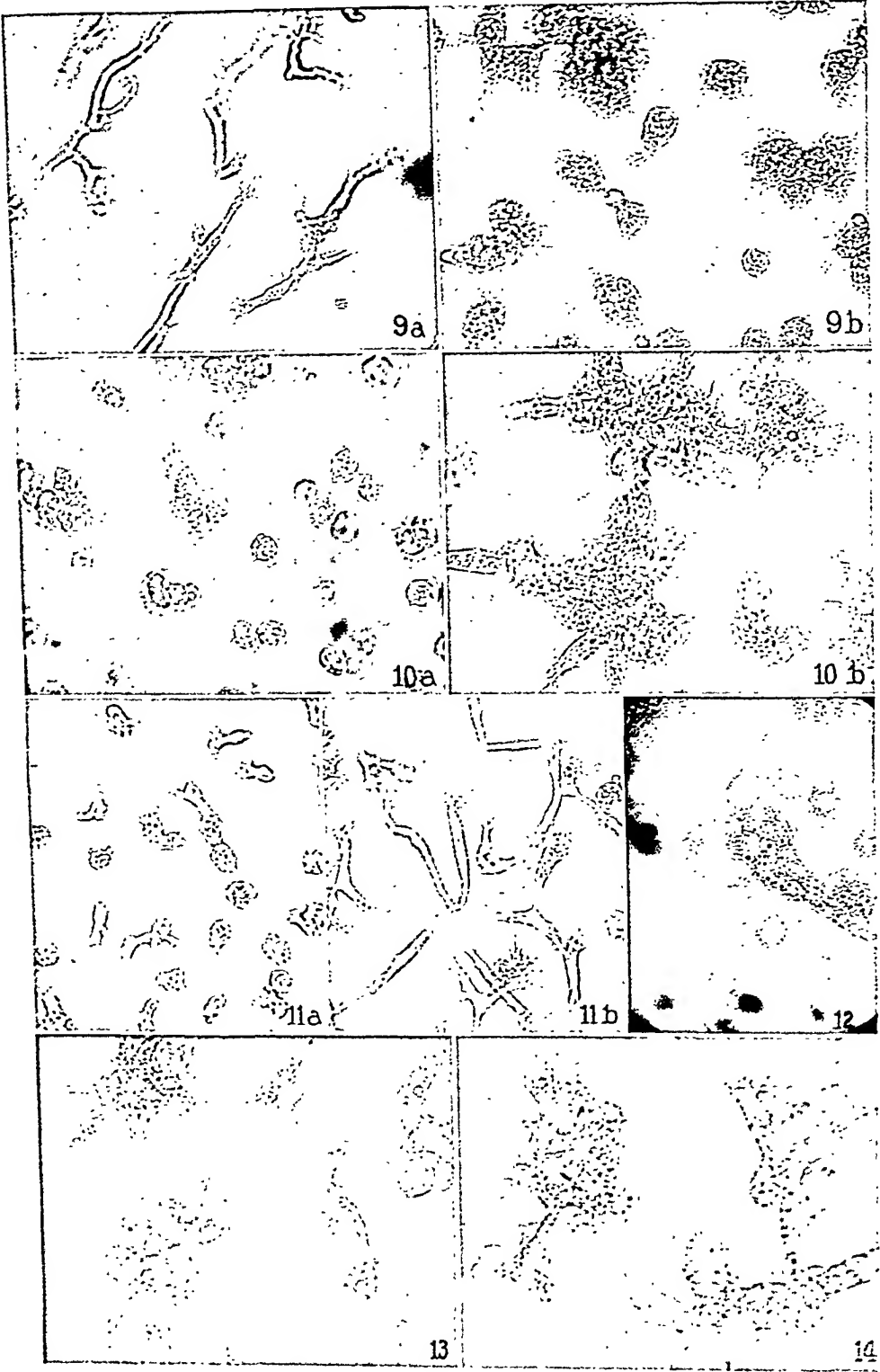
Finally, little is known as to the normal rate of blood protein destruction (18). While it is probably very slow as judged by endogenous protein metabolism in general, an abnormal acceleration must be considered as a possible factor in clinical hypoproteinemia.

EXPERIMENTAL

The purpose of the present experiments was to test, definitively if possible, the effect of low protein diet on the serum protein level of rats. The procedure differed from that employed by Kohman (6) and by Frisch, Mendel, and Peters (7) in three principal respects: many more animals were used, they were mature, and carrots, which may be a disturbing factor, were eliminated from the diet.

Material and Methods

Young but mature female white rats were used. They weighed, for the most part, 140 to 190 gm. The stock was that which has been inbred in this laboratory (19) for many years; the animals are vigorous and very satisfactory for observa-



Figures: Effect of d.p.s. on production of a response

and they ate well. From about the 3rd week on, the hair became discolored, brownish grey, and brittle and it fell out in patches of variable size. No dermatitis developed, however, and the bald areas soon developed an abundant growth of delicate snow-white hair. There were none of the usual evidences of vitamin deficiency; the eyes, feet, and skin remained normal. There was no rhinitis or diarrhea. Only one rat in the whole group of 144 died during the course of the experiment, on the 140th day. No animal developed any signs of edema.

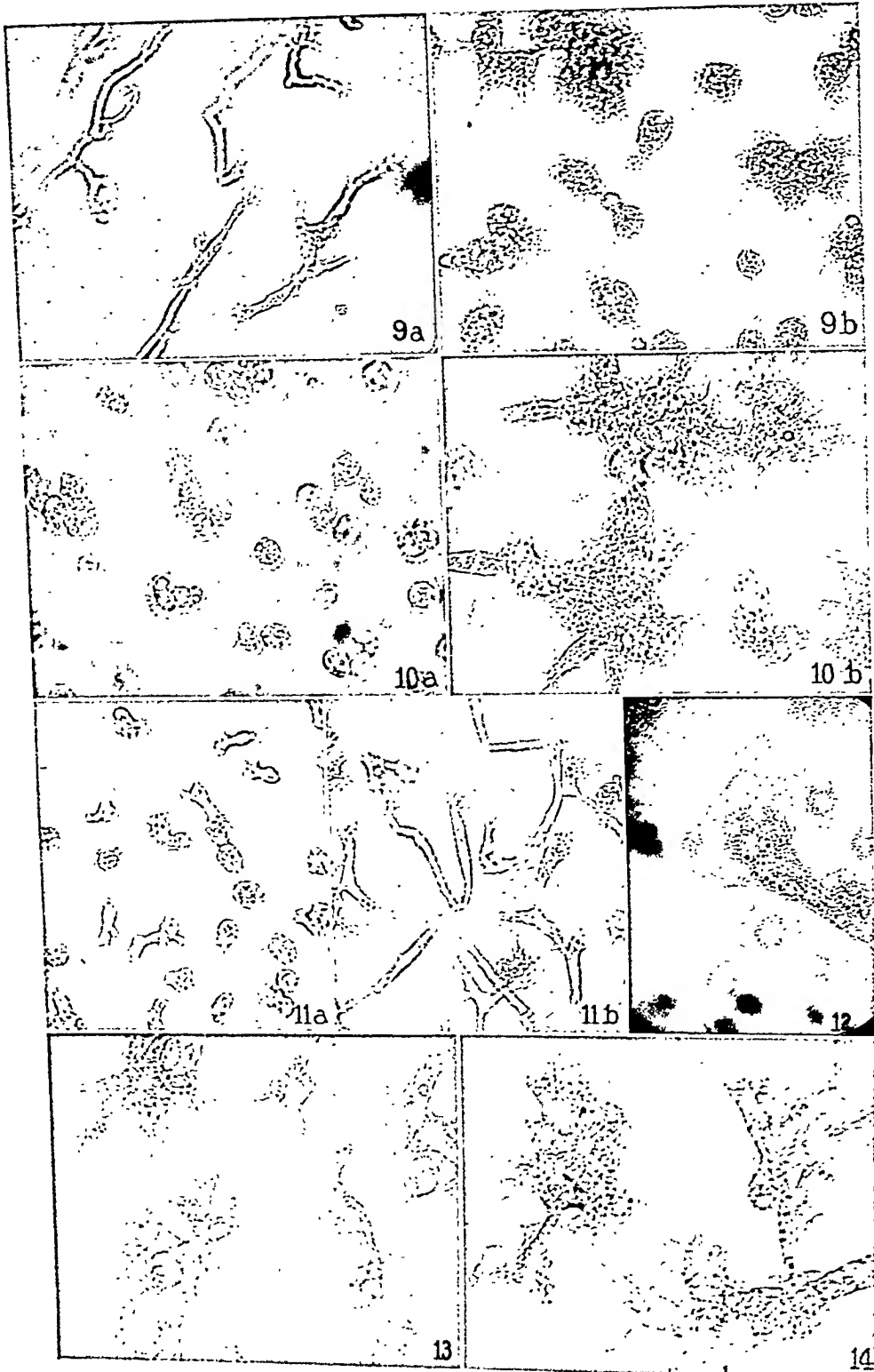
Examination of the organs (macroscopic) at various periods showed a progressive and obvious decrease in size but no apparent pathological lesions. Even after 20 weeks there was still a good deal of mesenteric fat. The muscles and subcutaneous tissues looked dry rather than moist.

The output of protein in the urine was determined in a group of eight rats which had been on the diet for 19 weeks. The method devised by Shevky and Stafford (22) was used. The average protein excretion per 12 hours per 100 sq. cm. of body surface was 0.1827 mg., a figure within the normal range (23).

That lack of protein in fact rendered the diet inadequate for maintenance is clear from the weight curves (Figs. 1 and 2). Fig. 2 shows the average loss at various intervals in terms of percentage of the initial weight which is taken as 100 per cent. With minor variations there was a steady decrease which at 21 weeks amounted to 26+ per cent.

Changes in the Serum Proteins.—As the rats lost weight the total blood volume was diminished. Figs. 3 *a* and 3 *b* show a definite correlation between weight and the total blood obtained by the present method of exsanguination. The serum volumes were not measured accurately so that no figures can be given as to their relation to body weight. It is evident, however, that the total serum proteins were markedly decreased. But the point of importance is not so much the total quantity of protein as the concentration.

Changes in Concentration of the Serum Proteins.—Fig. 4 and Table I show the values for serum protein concentration at various intervals during the course of the experiment. Each estimation was made on the pooled serum of a group of rats. The events can be divided into



(Baker: Effect of digestion products on monocytes)

two stages the first of which one may speak of as the initial drop. We have found when rats are put under a variety of conditions of low protein intake that there is a prompt fall in the concentration of the serum proteins amounting to approximately 10 per cent of the initial value. This fall may be manifest in a day or two or may extend over several days or even a week. The drop is at the expense of the serum albumin; the globulin remains essentially unchanged. Return

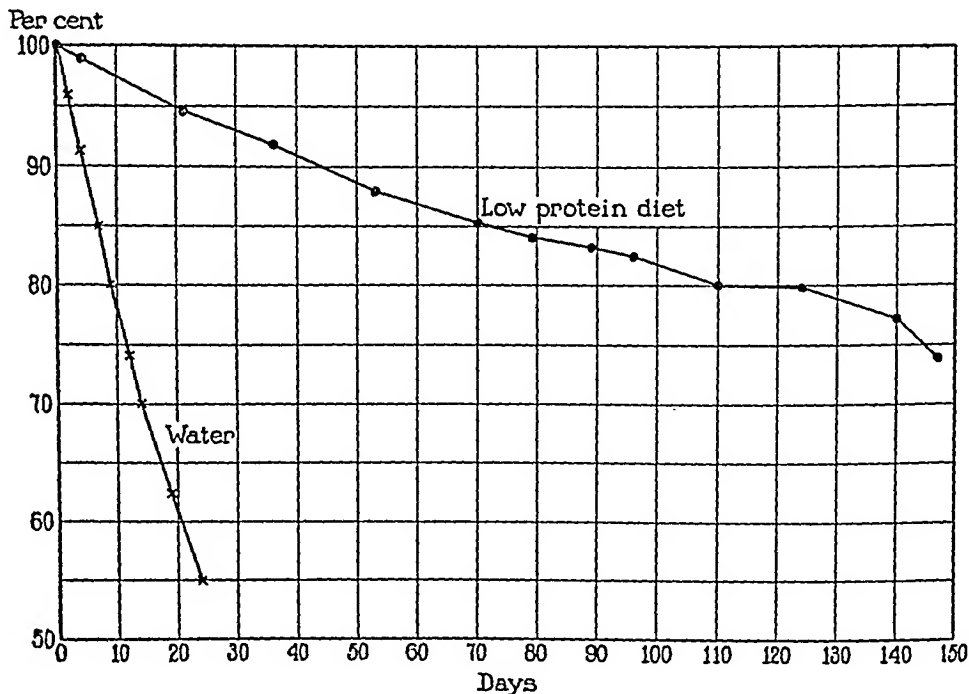


FIG. 2. Composite weight curves of rats on low protein diet and on fasting (water). Loss of weight at various times is expressed in percentage of initial weight (100 per cent).

to a diet containing adequate proteins is followed by an equally prompt restoration of the initial serum protein level. So rapid are these fluctuations and so small their extent, that we are inclined, tentatively at least, to regard them as of physiological and not of pathological significance. This view is reinforced by the stubborn resistance put up by the serum proteins to further depletion at the very time when readily available carbohydrate and fat are being exhausted, and body protein must be drawn upon as a source of energy, as in fasting (see

THE EFFECT OF RESTRICTION OF PROTEIN INTAKE ON THE SERUM PROTEIN CONCENTRATION OF THE RAT*

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Diminution of the plasma proteins which has been found to occur so frequently in certain types of Bright's disease and in various states of undernutrition is attributed by most students of the subject either to loss of protein in the urine or to lack of protein in the diet. In some cases it is believed that both loss and lack may play a part. The current views are clearly stated by Peters and Van Slyke (1) in their recent monograph, by McCann (2), and by Peters (3) in a long series of papers one of which deals especially with the status of the blood proteins in nephritis. The studies which have led up to present concepts of hypoproteinemia have been exposed so often in the recent literature that detailed review now would serve no useful purpose. We wish, however, to analyze critically the evidence for the loss and lack theory and to point out, as some writers have already suggested, that additional factors may play a part.

The arguments usually advanced in support of the loss and lack theory are the following.

1. There is an undoubted association between hypoproteinemia and the types of Bright's disease in which large quantities of protein are lost in the urine. This observation, confirmed every day in the clinic, certainly offers an apparent explanation of lowered blood proteins, and as Peters (3) points out "a patient of average size losing as much as 16 grams of protein a day . . . would excrete all the albumin of his serum in the course of a week or two."

2. The finding of hypoproteinemia without proteinuria in various states of malnutrition together with clinical improvement and rise of blood proteins after the patient has been placed on an adequate diet, high in protein, has been interpreted as indicating that lack of protein intake is the cause of the low blood proteins.

* Supported by a grant from the Rockefeller Fluid Research Fund.

two stages the first of which one may speak of as the initial drop. We have found when rats are put under a variety of conditions of low protein intake that there is a prompt fall in the concentration of the serum proteins amounting to approximately 10 per cent of the initial value. This fall may be manifest in a day or two or may extend over several days or even a week. The drop is at the expense of the serum albumin; the globulin remains essentially unchanged. Return

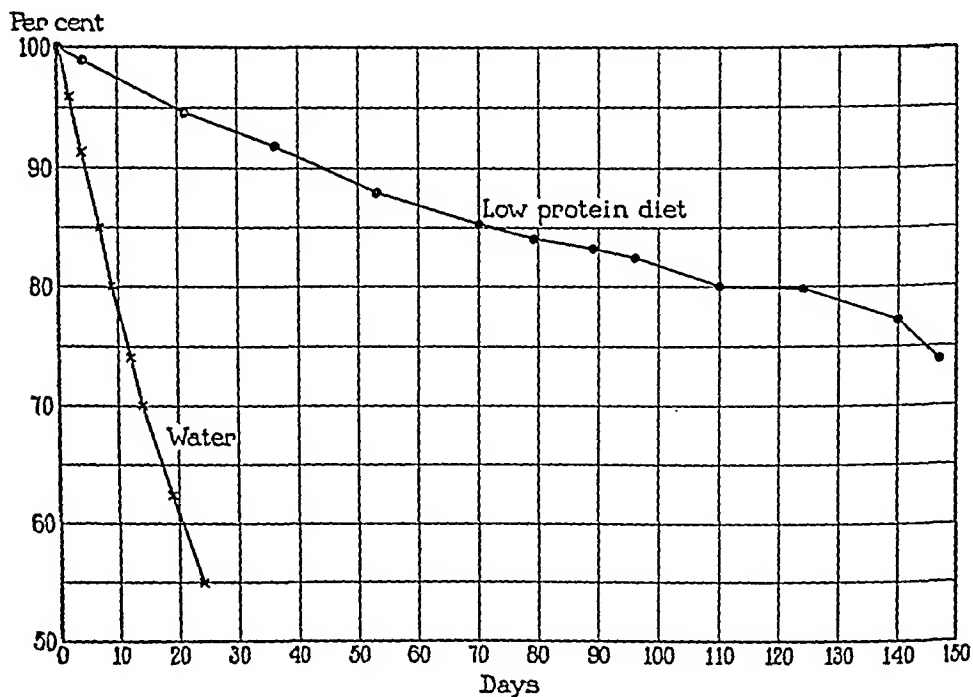


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part in the production of hypoproteinemia. The question may be raised, however, whether such a simple concept fits all the facts of clinical and experimental experience and whether one must not postulate in addition some injury to, defect of, or inadequacy of the blood protein-forming mechanism as an accessory if not a primary difficulty; in point of fact, the evidence for the loss and lack theory when inspected from this angle reveals a number of serious breaches which will next be analyzed.

While lowering of blood proteins, as we pointed out above, has resulted from experimental withdrawals of plasma, the procedure has always been an extreme one. In Leiter's experiments a large portion of the dogs' plasma was abstracted day after day, and Fishberg's rabbits suffered similar losses. Despite these drastic procedures which could hardly be paralleled in man under any natural conditions, the animals exhibited an amazing ability rapidly to regenerate their blood proteins. Whipple's dogs, for example, even when fasted after the bleeding, had built up their proteins to a normal level in 14 days, and Fishberg's Rabbit 4, although deprived of approximately half its blood each day for 18 days, maintained its plasma proteins during the last 10 days of the experiment at a nearly constant level of about 4 gm. per cent. It may be estimated from the protocol that during the 18 day period this rabbit regenerated the total amount of its blood proteins six times. Barnett, Jones, and Cohn (13), in this clinic have also shown that dogs may maintain a normal blood protein level in the face of daily large bleedings, although the procedure in this case was less formidable than in the plasmapheresis experiments of Whipple and of Leiter. It seems clear, then, that protein loss in order to be effective in lowering blood proteins must be extreme unless there is in addition some impediment to the remarkable regenerative mechanism which normally comes promptly into play.

Concerning the experimental results of low protein feeding (including fasting) in animals, the observations of Kohman and of Frisch, Mendel, and Peters have already been mentioned. They ascribed their findings purely to a lack of protein in the diet, but inasmuch as they dealt with young (60 gm.) rats which as a result of the treatment suffered a profound interference with their growth, another interpretation, namely that the protein-building mechanism was disturbed as a result of inanition, would have to be considered. This question is raised because other experiments reported in the literature seem to be at variance with the above observations. There are, for example, a good many papers dealing with the effects of fasting on the blood proteins. Hanson (14), working with rabbits, followed the plasma proteins during alternate periods of feeding and of fasting. The animals went without food for as long as 6 days. In no case was there any drop in the blood proteins. Hanson also reviews the older literature and refers to similar results in fasted dogs. Burkhardt (15) years ago found the total proteins only

this time on, however, over a period of 20 weeks—an interval which corresponds roughly to 10 years of a man's life (24)—there was no further significant drop in total serum protein. At 10 days a value of 5.35 gm. per cent was obtained, at 147 days a value of 5.47. Looking at it in another way, in order to smooth out minor variations, an average of all values from the 10th to the 70th days was 5.53 gm. per cent; from the 70th day to the end of the experiment 5.44 gm. per cent, a decrease of 1.6 per cent. Such a difference, if significant at all, is obviously negligible compared to decreases of serum proteins to 40 to 60 per cent of normal such as frequently have been found in

TABLE I
Concentration of Serum Proteins of Rats on Low Protein Diet

Length of time on low protein diet	No. of rats (pooled serum)	Serum protein
<i>days</i>		<i>gm. per cent</i>
Controls	70	6.25
2	30	5.82
10	30	5.35
20	30	5.68
28	6	5.70
56	6	5.48
91	8	5.52
98	6	5.33
112	6	5.93
126	6	5.30
133	6	5.31
140	5	5.14
147	5	5.47

human nutritional hypoproteinemias. Furthermore, partition of the serum from the last lot of rats, killed on the 147th day, into albumin and globulin showed again that the latter was within normal range and that the albumin was decreased just as was found in the case of the initial drop.

A group of five rats which had been on the low protein diet for 140 days were then placed on the control diet (casein 16 per cent). There was a rapid gain in weight (see Fig. 5) and after 6 days, when the animals were killed, the total serum protein had risen to 6.19 gm. per cent. This rise was found to be due to a restoration of the albumin fraction.

stress the benefit of high protein feeding in malnourished nephritics (Peters *et al.*, McCann, etc.) and while such feeding doubtless often improves their general condition it must be admitted that there is no adequate documentary evidence to show that such high feeding of protein specifically raises the blood proteins. Such slight changes as have been described may be found in subjects on a constant and even inadequate diet. Similar considerations apply to instances of hypoproteinemia in malnourished subjects without proteinuria. First of all, only a small percentage of malnourished patients show any marked lowering of the blood proteins and when they do there are usually other obvious factors aside from lack of protein in the diet, such as infection and the generally poor hygienic conditions of famine. Furthermore, the blood proteins may, on occasion, be unaccountably low in people who are not malnourished and who have not suffered from deficiency of protein in the diet. Youmans and Bell (17), for example, report curious instances of seasonal edema with low plasma proteins in subjects who were well nourished and who had no cardiac or renal disease. And, finally, the rise of blood proteins which gradually follows the exhibition of adequate diets in malnourished patients can, perhaps, be as well explained by a restoration of a debilitated blood protein-forming mechanism as a result of elimination of infection and general malnutrition as by a specific effect of protein. Bruckman, D'Esopo, and Peters (5), for example, report the effects of high protein feeding in a malnourished diabetic (their Table 1). Between Sept. 16 and Oct. 22 the serum proteins rose from 5.18 to 5.93, an increase in the total serum proteins of only about 16 gm., although during the period of observation there was a gain in weight of nearly 6 kilos. Clearly simple protein lack was not the cause of the low proteins since we know that a man can readily regenerate 16 gm. of plasma protein in 1 day whereas in this case even though nitrogen was being stored in large amounts it took 36 days to regenerate this amount. The only possible interpretation is that as the general nutrition improved and as she got rid of a severe infection of the hand which was present at the start the protein-forming mechanism gradually recuperated. Even when her condition was at its worst 16 gm. of protein could certainly have been diverted from other body stores to be reformed into blood proteins had the blood protein-regenerating mechanism been functioning properly.

The foregoing analysis forces one to the conclusion that the lack and loss theory is inadequate to explain the observed facts in all instances of hypoproteinemia. In normal animals there exists a tremendous capacity rapidly to regenerate blood proteins from the body stores of protein even during fasting, a capacity which obviously would hardly be taxed in many clinical instances of hypoproteinemia were there not some interference with its normal functioning. The evidence suggests that part at least of the difficulty which leads to lowering of the blood proteins is an impairment of the blood protein-

Finally, in order to reduce the problem of low protein intake to the simplest terms, all food was withheld from another group of rats. Water was allowed *ad libitum*. Aside from rapid loss of weight (see Fig. 2) the animals remained in excellent condition for periods of time up to 3 weeks depending upon the extent of the fat stores at the beginning of the fasting period. There were no signs of vitamin deficiency and the most striking finding at autopsy was a decrease in the mesenteric and retroperitoneal fat. Determinations of plasma or of serum proteins were made at various intervals (Fig. 6). Just as with the low protein diet there was an initial drop after which no significant decrease occurred despite the rapid loss of weight. The results are therefore in

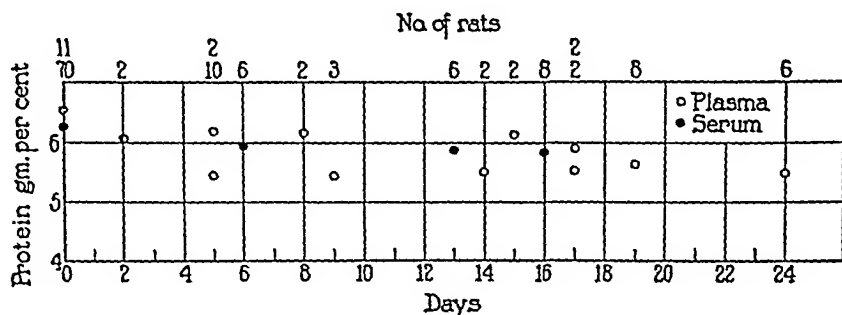
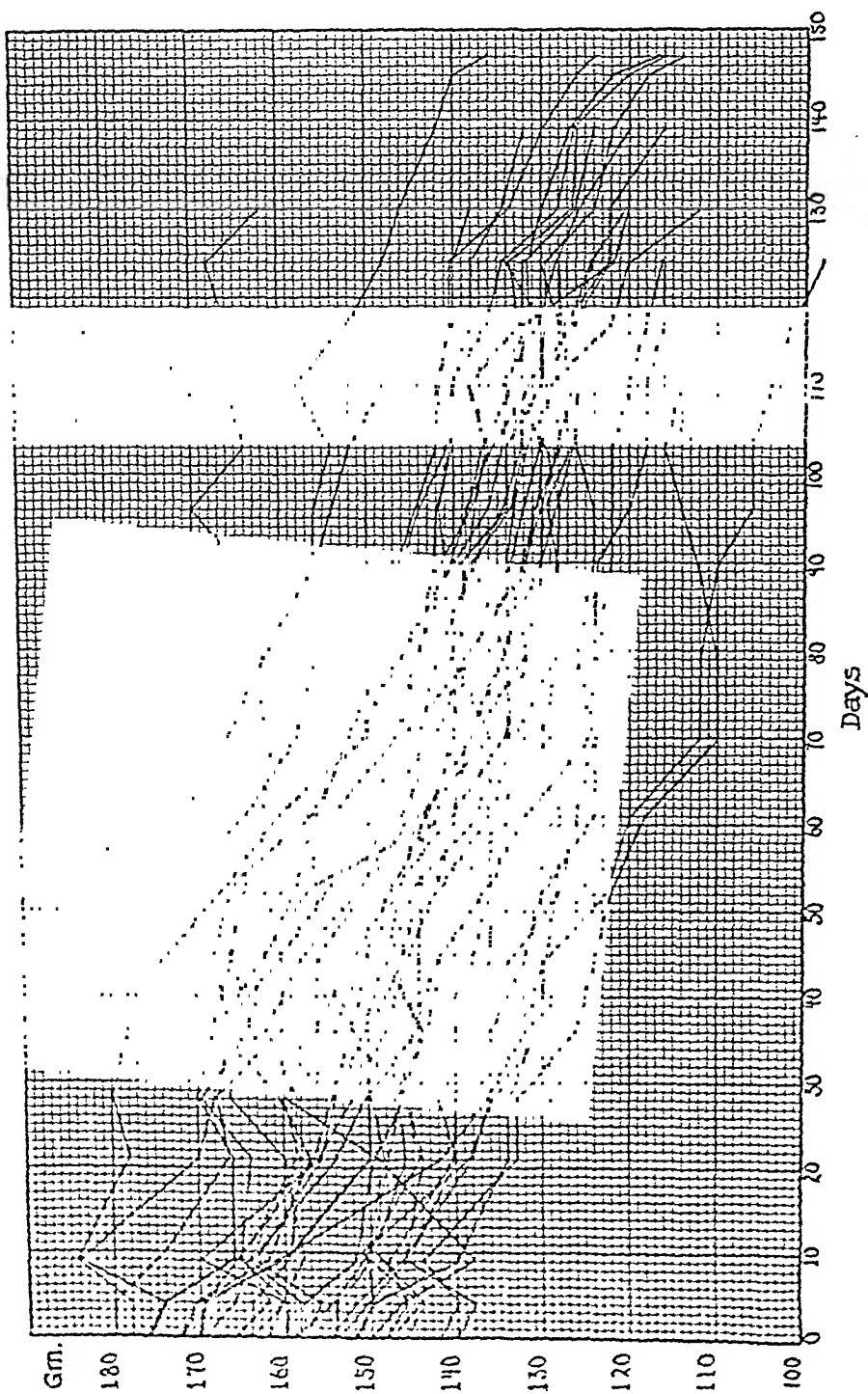


FIG. 6. Concentration of plasma or serum proteins from rats on water alone. Each dot indicates the value obtained from pooled serum or plasma from a number of animals.

harmony with those obtained in the long time low protein diet observations.

DISCUSSION

It has been shown, in brief, that aside from the initial drop, which is probably a physiological phenomenon, a low protein diet fed over a period of 21 weeks led to no significant decrease in concentration of total serum proteins despite marked loss of body weight. The blood volume and the total serum proteins were decreased, roughly, in proportion to weight loss. To what extent the serum proteins are broken down and to what extent body protein is diverted to their restoration so that their concentration can be maintained is not revealed by the present experiments. The exact nature of the mechanism requires special analysis.



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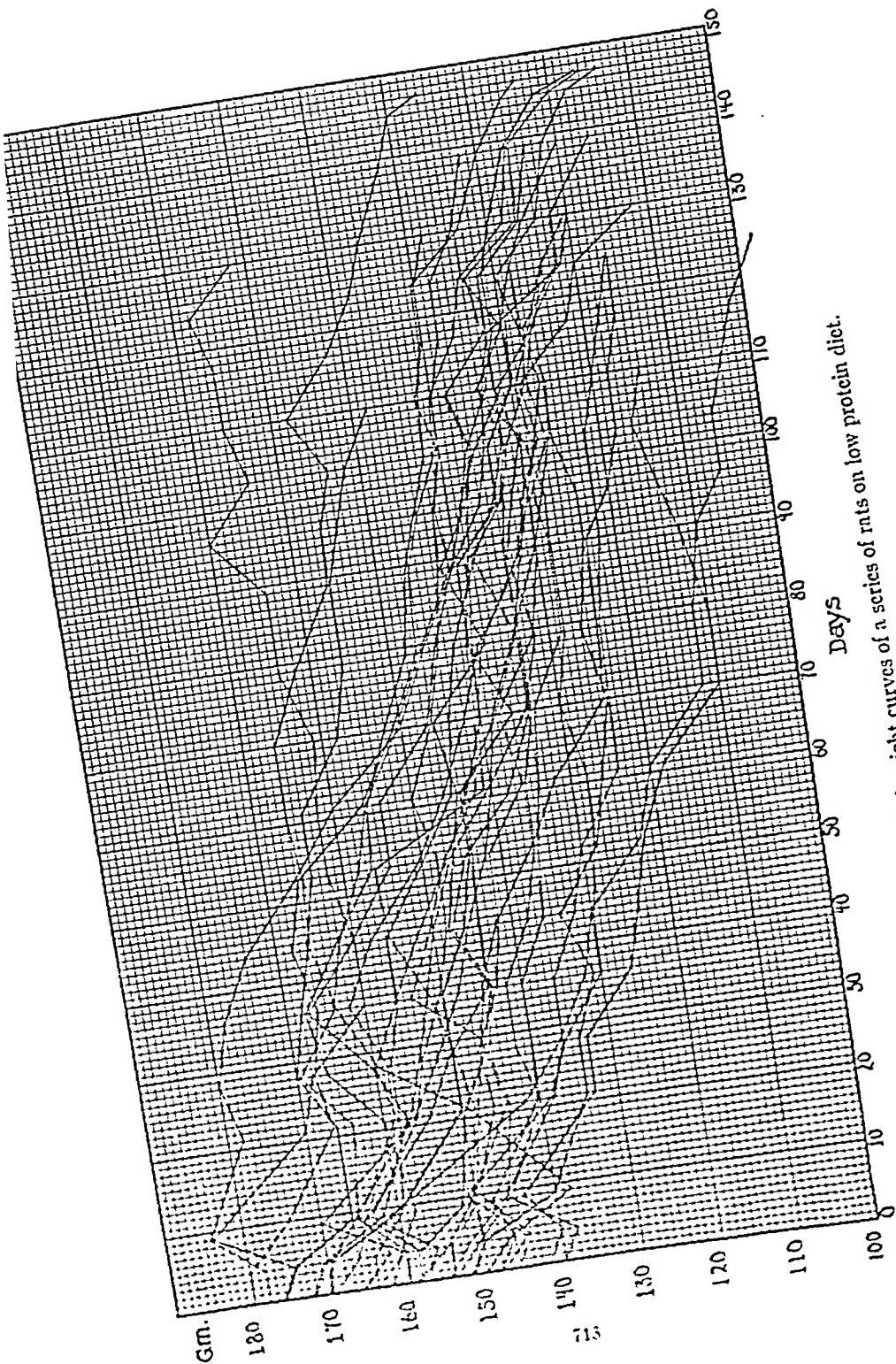


FIG. 1. Individual weight curves of a series of rats on low protein diet.

It was with this idea that a series of experiments was undertaken. We have been able to show that the variations in antigenic volume, as the result of agglutination, can be demonstrated by the procedures outlined. In the present paper we note the procedure and the results; discussion of the findings and interpretation of the facts will be left to another paper.

EXPERIMENTAL

Most of the experiments were of a similar nature and the same procedure was rigidly followed. Bacterial antigens were prepared from young cultures grown on plain agar, and the organisms were suspended in 0.9 per cent sodium chloride. The number of organisms was always sufficient to give a volume of 5 c.mm. or more per unit after centrifugation at 2900–3000 R.P.M. for 1 hour in the maxiforce centrifuge. Antigens of such concentrations contained two to three times as many organisms as those employed in the usual agglutination tests. Special pains were taken thoroughly to mix and to distribute the antigen. The same pipette was used throughout the series and where the entire contents of the pipette were not used the measurement was always between the same points.

The immune sera were not specially prepared for these experiments. Those for the paratyphoid group of organisms were obtained by immunization of rabbits; for *B. abortus* the serum of cows naturally infected was used.

The method of determining differences in volume was as follows: Various dilutions of immune serum were added to capillary centrifuge tubes which had been previously calibrated.¹ An additional tube containing only salt solution and antigen was carried as a control. One unit of antigen was added to each tube and its contents mixed. All tubes were incubated 2 or 2½ hours, centrifuged in the maxiforce for 1 hour at a speed of 2900–3000 R.P.M., and, by means of a divided ocular and a stage micrometer, the length of column under a magnification of 16 diameters was determined. From this the volume was calculated.

It might be mentioned that neither the period of incubation, provided it was an hour or more, nor subsequent storage in the refrigerator affected the results.

Experiments 1 and 2 record the volumetric change of antigens of motile and non-motile organisms when subjected to the action of specific serum.

Experiment 1.—The growth from three agar slants of 24 hour cultures of *B. aertrycke* was suspended in 35 cc. of 0.9 per cent sodium chloride. Various concentrations of diluted antiserum in quantities of 2.5 cc. were placed in the capil-

¹ We are indebted to Dr. M. Kunitz of The Rockefeller Institute for calibration of the capillary centrifuge tubes.

below). The final explanation is not at hand and the matter requires further study.

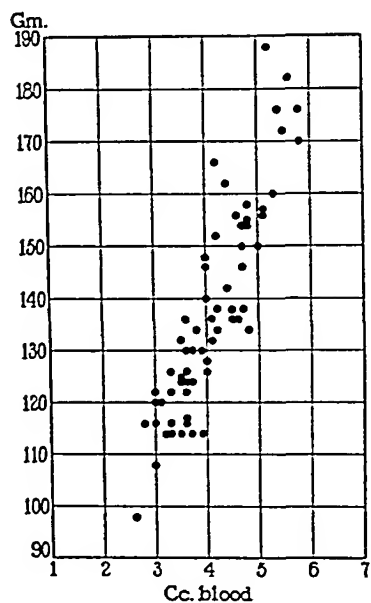


FIG. 3a

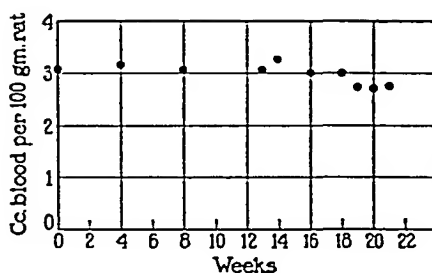


FIG. 3b

FIG. 3a. Relation of body weight to total blood volume as measured by exsanguination. Each dot refers to a different rat.

FIG. 3b. Cubic centimeters of whole blood per 100 gm. of rat. Averages of groups at various intervals.

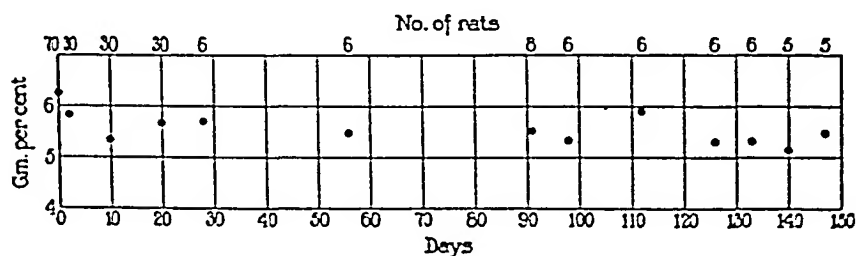


FIG. 4. Serum protein concentration of rats on low protein diet. Each dot gives the value obtained from pooled sera of a number of animals.

At any rate, in the present experiment this initial drop took place. It was evident by the 2nd day and obvious on the 10th day. From

was that of a cow naturally infected. 5 cc. of antigen and 5 cc. of diluted serum were used in this experiment. The results are given in Table II.

The measurements recorded in Tables I and II indicate that there is an increase in the volume of the antigen in the presence of immune serum. There is in general a correlation between volume and concentration of antibody.

It might be inferred that during agglutination various substances were precipitated, thus adding to the volume. The actual specificity of the change in bulk is open to question since it could be said that normal serum or immune serum freed from antibody by specific absorption might produce volumetric increase. The next series of

TABLE III

Volumetric Change in B. aertrycke in Normal and Immune Serum

Normal serum			Immune serum	
Dilution	Volume	Agglutination	Volume	Agglutination
	<i>c.mm.</i>		<i>c.mm.</i>	
1:20	5.28	+-	8.97	C
1:40	5.28	+-	8.09	C
1:80	5.28	-	7.39	C
1:160	5.28	-	7.21	C
1:320	5.28	-	6.86	C
Control, 0 serum	5.28	-		

experiments may be regarded as bearing on such points. It is typical of many in which volumetric changes in antigen in the presence of normal and immune serum are recorded.

Experiment 3.—Antigen from cultures of *B. aertrycke* was prepared in the usual manner and equal quantities were added to diluted normal rabbit serum and immune rabbit serum. Adequate controls were maintained. All tubes were incubated for 2½ hours and then centrifuged at the usual speed and the volume of the antigen determined. In Table III the results are recorded.

Experiments in which non-motile hog cholera bacilli were employed with both immune and normal serum produced similar results. The same was true with *B. abortus* except that the normal cow serum agglutinated the bacilli at low dilutions and had much the same effect

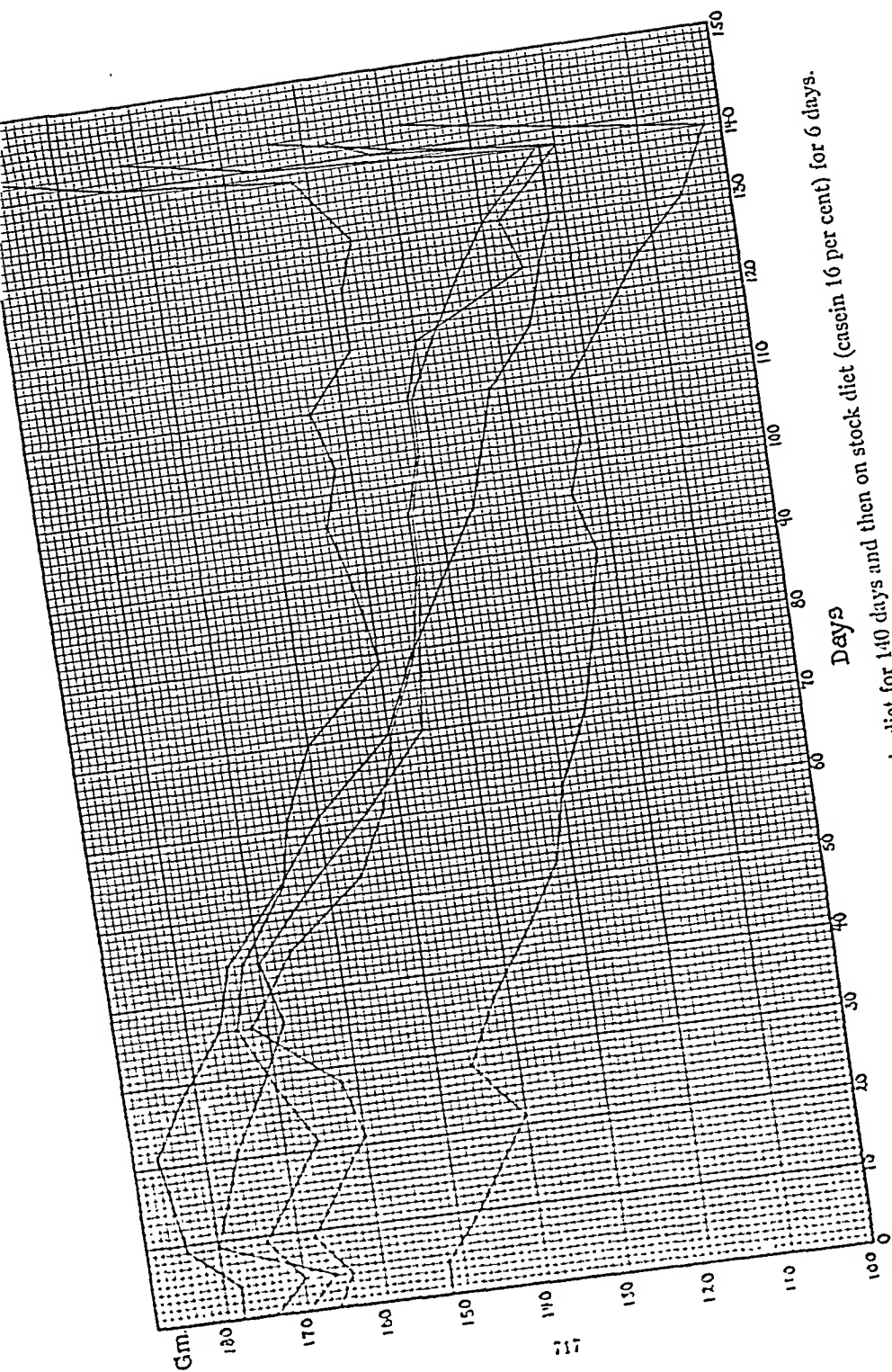


FIG. 5. Weight curves of five rats on low protein diet for 140 days and then on stock diet (casein 16 per cent) for 6 days.

There is then no readily soluble substance of an extraneous nature that might be considered the cause of the increase in antigenic volume.

Experiment 5.—*B. aertrycke* immune serum diluted 1:5 was twice absorbed with massive numbers of the specific organism. After the final centrifugation the effect of the clear supernatant on the volume of antigen was determined. Another portion of the same serum, not subjected to absorption, was used for control. The volumes under the given conditions were determined after incubation and centrifugation and are recorded in Table V.

While it is true that some increase in the volume of the antigen occurred in the lower dilution of the absorbed serum, nevertheless the differences between the measurements in this series and those in the

TABLE V

The Effect of Specific Agglutinin Absorption on Volumetric Change in B. aertrycke

Absorbed serum			Unabsorbed serum	
Dilution	Volume	Agglutination	Volume	Agglutination
	<i>c.mm.</i>		<i>c.mm.</i>	
1:20	6.86	+++		
1:40	6.86	++	9.68	C
1:80	6.51	+	8.62	C
1:160	5.63	+-	8.10	++
1:320	5.63	—	7.57	++
				++
1:640	5.80	—	7.39	+++
Control, 0 serum	5.80	—	5.80	—

other are sharp. The absorbed serum behaved like a weak agglutinin, and in fact can be so regarded, since all the agglutinin was not removed by absorption. The same effects were noted with *B. abortus* in a specifically absorbed serum.

DISCUSSION

We have already stated that the aim of the experiments was to determine whether or not there was a detectable increase in the volume of antigen as the result of agglutination. The experiments indicate that by the procedure described, differences in antigenic volume can be measured. As a general proposition it is true that increase in

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Our results do not agree with those of Kohman (6) and of Frisch, Mendel, and Peters (7), who, as we pointed out, obtained a marked lowering of plasma protein concentration with low protein diets. The explanation of this discrepancy lies, perhaps, in the fact that carrots were one of the principal features of the diet used by these investigators; we have found that a diet of carrots alone leads to a definite fall in serum protein concentration (25). In Frisch's experiments, furthermore, the repeated withdrawal of blood for the tests may have played a part in lowering the plasma proteins.

The present observations have, of course, no direct bearing on the nutritional hypoproteinemia of man. They suggest, however, the need of a reassessment of the various possible etiological factors with emphasis on influences other than protein deficiency *per se*. The effects of inadequate caloric intake, vitamin deficiency, excess of certain foods such as carrots and turnips, and infection are especially to be considered.

CONCLUSIONS

Rats placed on a low protein diet for 21 weeks, in spite of marked loss of body weight, showed no significant decrease of serum protein concentration aside from an initial (physiological ?) drop.

I am indebted to Dr. R. B. Cohn for assistance with some of the plasma protein determinations, and to Dr. Eloise Jamieson for the measurement of the albumin globulin ratios.

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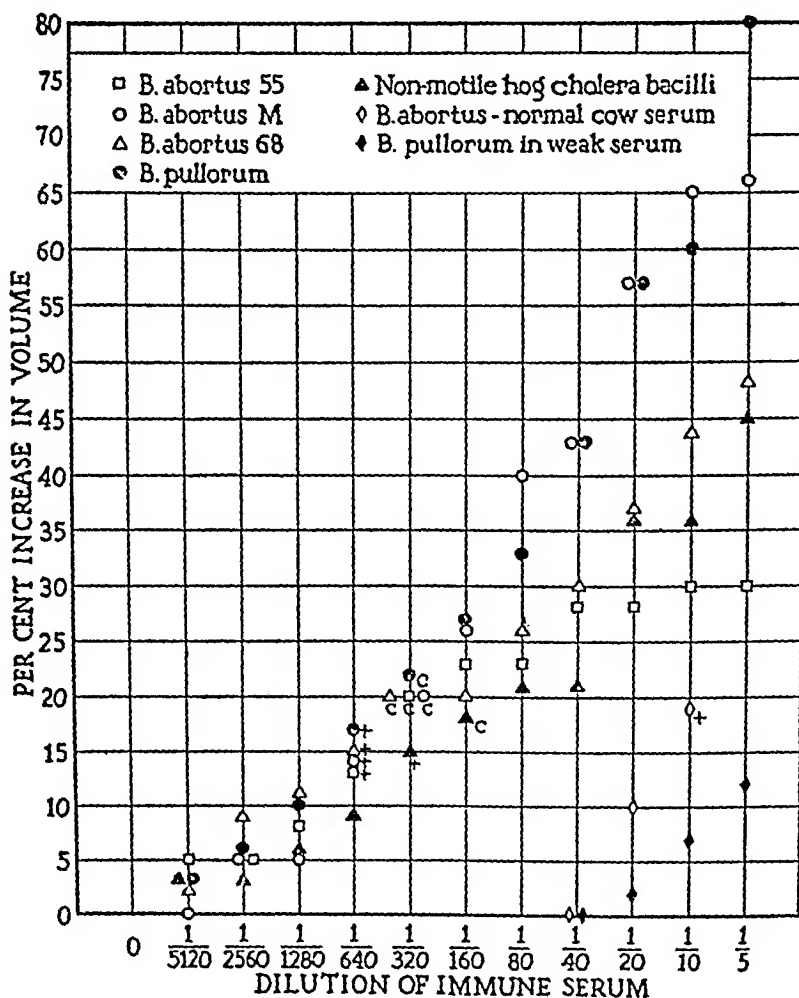
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It will be seen that as the concentration of the immune serum was increased, the changes in antigenic volume became more marked, so that to cite the extreme instance, *B. pullorum* increased by volume 80 per cent in the most concentrated serum dilution. In general, how-



TEXT-FIG. 1. Percentage increase in volume of non-motile organisms in immune sera.

+ indicates agglutination but with the supernatant not entirely clear.

C indicates complete macroscopic agglutination.

ever, the increase lay between 30 and 65 per cent. The gain in volume was a gradual one, progressing upward toward maximum concentration but as a rule showing no tendency to become constant in the most concentrated serum dilutions.

CHANGES IN BACTERIAL VOLUME AS THE RESULT OF SPECIFIC AGGLUTINATION

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The fact that during specific bacterial agglutination globulin from immune serum is deposited upon the bacterial surface, is well shown by the experiments of Northrop and De Kruif (1), Shibley (2), and Eagle (3). The more recent work of Heidelberger and Kendall (4) indicates that the precipitate resulting from the mixture of polysaccharide of the pneumococcus and a purified homologous antibody, varies in its composition over a definite range. At the optimum reaction the precipitate is composed of 1 part antigen to 120 parts serum globulin. In both agglutination and precipitation, the optical effect (flocculation) may be said to represent deposition of globulin. In the case of agglutination, particles (bacteria) of microscopic size become coated with globulin, while in precipitation, molecules, or aggregates of molecules, are coated. That the two substances, agglutinin and precipitin, are of similar nature has been indicated by experiments of a different character. Arkwright (5) found that *B. coli*, mixed with an extract of another organism, promptly agglutinated when immune serum specific for the extracted organism was added. Jones (6), and Mudd and his coworkers (7) showed that collodion particles agglutinated if first coated with antigen and later mixed with precipitin specific for the antigen.

If it be true that bacteria in the presence of immune serum are coated with globulin, and the ratio of deposition of globulin is comparable to that found by Heidelberger and Kendall in precipitation, bacteria should increase in size as the result of agglutination. It might be argued further that the increase in size would be within certain limits proportional to the quantity and titre of the immune serum employed in the reactions.

smallest increase at the $\frac{1}{2}$ level, as indicated by + in the text-figures, is 9 per cent and the greatest 20 per cent, while with complete clumping it varies between 12 and 27 per cent. The average, however, of 16 per cent at the $\frac{1}{2}$ point and 20 per cent at complete agglutination, strengthens the inference that, regardless of the quantity of serum employed, when the antigenic volume has been increased about 16 per cent nearly all the bacteria are agglutinated and when the increase is 20 per cent or more agglutination is complete.

It should be stated that only the first complete agglutinations (C) are recorded in both figures. To the right of these points the agglutination was always complete, whereas to the left of the points indicated by + the result was always less than $\frac{1}{2}$.

Thus far all the data have dealt with the fact that bacteria increase in size as the result of union with specific antibody. A number of explanations might account for the volumetric change. The increase might be considered as a measure of globulin deposition from the immune serum. The deposition of protein might be focal and tend to cushion the bacteria one from the other and increase the spaces between the cells thus altering the volume when the mixtures were centrifuged. On the other hand swelling of the organism might occur as result of the chemical change in the cell surface due to union with antibody.

It seemed possible to determine by experiment whether or not the deposition of protein from the immune serum was sufficient to account for the change in volume. The next series of experiments deals with this question.

It is known (2) that collodion particles, when first soaked with antigen and subsequently washed, will agglutinate in the presence of precipitin specific for the protein used in sensitization. Such material seemed to us to afford opportunity for direct comparisons between volume and protein adsorbed from the immune serum. In Experiment 1 the effect of agglutination on the volume of sensitized collodion particles is shown.

Experiment 1.—25 cc. of a heavy suspension of collodion particles was added to 12.5 cc. of a 2 per cent solution of crystallized egg albumin. The mixture was incubated 2 hours, centrifuged, and the particles resuspended in sodium chloride. The centrifugation and washing were twice repeated. After the final washing,

lary tubes and 2.5 cc. of antigen added. The tubes were incubated for 2 hours, refrigerated 4 hours, and centrifuged for 1 hour at 2900-3000 R.P.M. A tube containing one unit of antigen and an equal volume of sodium chloride served as a control. As a further control smaller quantities of antigen were tested with the

TABLE I
Volumetric Change in B. aertrycke as the Result of Agglutination

Serum dilution	Volume	Result of regular agglutination tests
	<i>c.mm.</i>	
1:80	9.86	C
1:160	9.33	C
1:320	8.8	++
		++
1:640	8.1	+++
1:1280	7.92	++
1:2560	7.57	+
1:5120	7.74	+-
Control, 0 serum	7.57	-

TABLE II
Volumetric Change in B. abortus as the Result of Agglutination

Dilution of serum	Volume	Result of regular agglutination test
	<i>c.mm.</i>	
1:5	7.39	C
1:10	7.57	C
1:20	7.39	C
1:40	6.86	C
1:80	6.68	C
1:160	6.68	C
1:320	6.51	C
1:640	6.16	C
1:1280	6.16	++
		++
1:2560	5.98	++
1:5120	5.46	+-
Control, 0 serum	5.28	-

immune serum in the regular manner and the results recorded after 2 hours' incubation and 20 hours' refrigeration. The results are given in Table I.

Experiment 2.—The experiment was similar to Experiment 1 except that a heavy suspension of *B. abortus* was employed as antigen and the immune serum

Experiment 2.—Both sets of data were obtained from identical, independent procedures. Heavy suspensions of collodion particles were mixed with an equal volume of 3 per cent crystallized egg albumin, incubated 2 hours, and centrifuged. The particles were then washed in three changes of distilled water, resuspended in 0.4 per cent sodium chloride, and distributed in quantities of 5 and 10 cc. An equal quantity of 1:10 dilution of anti-egg albumin serum was added to some of the tubes but not to the controls. All were incubated 2 hours, refrigerated overnight, and the volume of the particles in one of the agglutinated series and one of the controls was determined after centrifugation for $1\frac{1}{2}$ hours in the maxiforce at 2900–3000 R.P.M.

The particles in other tubes were washed in three changes of sodium chloride and resuspended in a small volume of distilled water and sufficient 3.6 normal sodium hydroxide added to hydrolyze the protein. Hydrolysis was carried on at

TABLE II

Volumetric Change in Sensitized Agglutinated Collodion Particles Compared with Volume of Protein Absorbed during Agglutination

		Volume per unit	Protein per unit
Experiment A	Sensitized particles	14.14 c.mm.	0*
	" and agglutinated particles	15.48 "	4.85 c.mm. 4.7 "
Experiment B	Sensitized particles	10.20 "	0*
	" and agglutinated particles	11.44 "	4.72 c.mm.

* Little color on addition phenol indicator.

40°C. for $\frac{1}{4}$ hour. All tubes were then centrifuged and the protein content of the clear supernatant measured by the colorimetric method of Greenburg (3). The results of two experiments are given in Table II.

The results of both experiments closely resemble each other and indicate that sensitized collodion particles, when agglutinated specifically, gain in volume but little although the actual protein adsorbed from the immune serum is considerable. The sensitized but unagglutinated particles in both experiments totaled in volume 24.3 c.mm. The same quantity of agglutinated sensitized particles had a volume of 26.9 c.mm., a difference of 2.6 c.mm. The same units of agglutinated sensitized particles had taken up nearly 9.6 c.mm. of protein.

The experiment indicates that sensitized collodion particles actually

as a weak agglutinin with the result that there was an increase in volume at the lowest dilutions (1:5 and 1:10) in which the organisms were agglutinated.

The question of whether the phenomenon of volume increase might be caused by an addition of matter extraneous to antigen-antibody union was considered in Experiment 4.

Experiment 4.—Equal quantities of *B. abortus* suspension were mixed with diluted immune serum (cow). All tubes were incubated and centrifuged. The volumes were determined and all the supernatant was withdrawn and replaced

TABLE IV

Volume Change in B. abortus Exposed to Immune (Cow) Serum and Subsequently Washed Twice

Dilution	Volume in original mixture	After first washing in NaCl	After second washing in NaCl	Agglutination
	<i>c.mm.</i>			
1:5	7.39	7.39	7.39	C
1:10	7.57	7.39	7.39	C
1:20	7.39	7.57	7.39	C
1:40	6.86	7.21	6.86	C
1:80	6.68	7.21	6.34	C
1:160	6.68	7.04	6.16	C
1:320	6.52	6.69	6.69	C
1:640	6.16	6.33	5.46	C
1:1280	6.16	5.80	5.28	++
				++
1:2560	5.98	5.28	4.93	++
1:5120	5.46	5.01	4.75	+—
Control, 0 serum	5.28	5.01	4.75	—

with 10 cc. of 0.9 per cent sodium chloride solution. After thorough mixing the tubes were again centrifuged and the volumes redetermined. The washing and centrifugation were again repeated and the results recorded as indicated in Table IV.

The same results were obtained in a similar experiment when *B. aertrycke* and its immune serum were mixed and the agglutinated bacteria washed in sodium chloride solution. Washing and repeated centrifugation only served to accentuate more sharply the volumetric differences between agglutinated and unagglutinated organisms.

terest in other respects. It is possible to measure directly how much protein is taken up by a large number of organisms in the presence of agglutinin and from this, provided the number of organisms is known, to calculate¹ the quantity of protein absorbed per bacterium. The approximate thickness of the deposited layer of protein, provided it is spread over the whole surface, can be learned and the ratio of this layer to the diameter of a molecule of protein calculated. These matters are considered in the discussion.

TABLE III
Volumetric Change and Protein Absorption as the Result of Agglutination

	Approximate area of one organism	Approximate No. of bacteria employed in the determinations	Volume 100 cc. antigen			Protein per 100 cc. antigen		
			Unagglutinated control	Agglutinated	Difference	Unagglutinated control	Agglutinated	Difference
<i>B. abortus</i> in sodium chloride solution + 1:20 immune serum	2.86 x 10 ⁻⁶ mm. ² Cylinders	9.7 x 10 ¹¹	c.mm. 183	c.mm. 249.0	c.mm. 66.0	c.mm. 38.5	c.mm. 49.2	c.mm. 10.7
<i>B. aertrycke</i> in sodium chloride solution + 1:40 immune serum	3.4 x 10 ⁻⁶ mm. ² Cylinders	7.8 x 10 ¹⁰	102	137.2	35.2	15.9	30.15	14.25

DISCUSSION

It is true that bacterial suspensions when mixed with specific agglutinin increase in cubic content. The increase is more or less regular, beginning in the lowest concentrations of immune serum and progressing as the serum concentration increases. It has been shown that in the instance studied when the volumetric increase is about 20 per cent the agglutination is complete. The phenomenon occurs even when

¹ We wish to thank Dr. M. Kunitz for his cooperation and suggestions in the matter of the calculations.

volume appears to follow concentration of immune serum. The effect of immune serum is specific since antigenic volume is not appreciably affected in normal serum nor in specific serum from which agglutinins have been removed by previous adsorption with antigen. Experiments in which antigenic volume was measured after agglutination and the packed organisms subsequently twice washed further indicate that the increase is a persistent one and cannot be attributed to the precipitation of matter extraneous to the antigen-antibody union.

Further discussion of the fact that there occurs, as the result of specific agglutination, an increase in antigenic volume will be left for a later paper.

SUMMARY

Measurements indicate that bacterial antigens increase in volume as the result of specific agglutination. There is a general parallelism between the increase in antigenic volume and the concentration of the immune serum. The phenomenon is specific. There is no increase with normal serum; with absorbed serum the increase is slight and it can be correlated with the presence of unabsorbed antibody. The effect is enduring as shown by volumetric determinations upon repeatedly washed, agglutinated bacteria.

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packing should be similar to that of unagglutinated organisms. The ratio of one volume to the other will then be that of the adsorbed protein. Ample evidence has been advanced to show that this is not true. In experiments previously reported by Jones (4) it was shown that when collodion particles were successively sensitized with five different antigens they could be agglutinated by any one of the precipitins specific for the antigenic substances. The phenomenon was explained by assuming that the whole particle surface was not covered by one antigen but that adsorption occurred on small areas. On these small areas the immune serum proteins were deposited and particle agglutination resulted. Much the same sort of explanation might be advanced in the case of bacterial agglutination. If only portions of the surface are antigenic, then it is conceivable that the globulin from the specific serum is deposited, not over the whole surface, but over the antigenic areas. Should this be true, the antigenic areas overlaid with serum proteins would protrude above the surrounding structure and when the suspensions were centrifuged would serve as pads preventing maximum packing of the bacilli. The apparent increase in volume then might be expressed as the function of the adsorbed protein in preventing maximum packing of the organisms. The fact that the apparent volume increase is a progressive one beginning at the greatest serum dilution and extending to the least suggests that the phenomenon depends indirectly on adsorption of protein. This fits the theory.

Little in the way of direct evidence can be advanced to substantiate the view that unencapsulated bacteria swell as the result of antibody union. In a number of experiments suspensions of paratyphoid bacilli were sensitized with serum diluted with distilled water and examined both in the hanging drop and in fixed and stained preparations. Comparisons with organisms treated with normal serum failed to show differences in size that were readily apparent by microscopical methods.

SUMMARY

Findings are described which amplify those of a preceding paper in showing that bacteria increased in volume when treated with specific agglutinin. When the increase in volume approximated 20 per cent

THE SIGNIFICANCE OF CHANGES IN ANTIGENIC VOLUME AS THE RESULT OF SPECIFIC AGGLUTINATION

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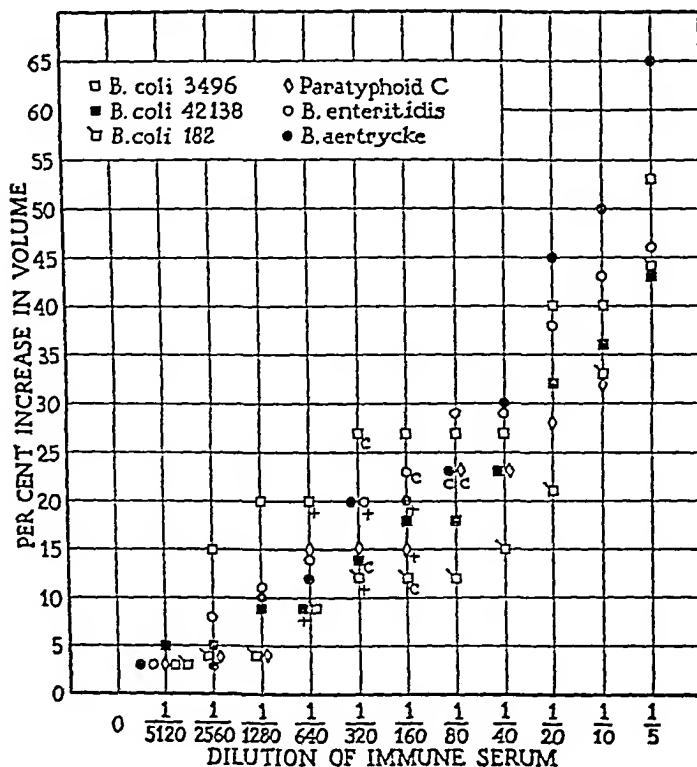
In our first paper (1) it was noted that bacteria increase in volume as the result of specific agglutination. The experiments indicated that the increase was a specific one due to the union of the antibody with the bacterial cell. The present paper provides further experimental data bearing on the general phenomena of bacterial agglutination together with a discussion of the significance of the findings.

EXPERIMENTAL

The first series of experiments were all of the same sort and consisted of volumetric measurements of various bacterial antigens in different concentrations of homologous antisera. The procedure was that already employed.

The data in Text-figs. 1 and 2 are expressive of actual measurements and were obtained as follows: A series of twelve capillary centrifuge tubes was set up. Eleven contained the diluted immune serum, and the twelfth salt solution equivalent in volume to the diluted serum. The same quantity of antigen was added to each tube and after incubation for 2 hours all tubes were centrifuged at 2900–3000 R.P.M. for 1½ hours in the maxiforce centrifuge and the quantity of sediment was measured. The volume of the sediment in the control tube (containing no serum) was taken as 100 per cent. The changes in bacterial volume in the tubes which contained immune serum were determined and expressed in per cent increase in volume. For purposes of convenience the per cent increase in volume has been plotted against dilution with the greatest dilution toward the left and the greatest concentration at the right. These figures in turn have been correlated with the effect of the same concentrations of immune serum in proportionately the same quantity of antigen, and the gross effects have been recorded after 2 hours' incubation and 18 hours' refrigeration. For greater simplicity the data have been divided. Text-fig. 1 deals with changes in volume of non-motile organisms and Text-fig. 2 with motile organisms.

The relation of percentage of volume increase to actual agglutination has much interest. Points indicated by + in both figures indicate that at this concentration of serum the agglutination is nearly complete (frequently expressed as $\frac{++}{+}$). Such points in the case of non-motile organisms are associated with 13 to 18 per cent increase in the



TEXT-FIG. 2. Percentage increase in volume of motile organisms in immune sera.

bulk of the bacteria. When all the organisms are agglutinated, as indicated by the symbol C, the volume has increased from 17 to 22 per cent. One can say that when the volume of the non-motile organisms has been increased about 16 per cent nearly all are agglutinated and when the increase approaches 20 per cent, agglutination is complete. With the motile bacilli the results are more variable since the

Eagles and McClean (2, 3), failed to provide adequate descriptions of their methods. For instance, they give minute details about the method of obtaining the "cell-free" kidney extract but say nothing of the manner in which the rabbit serum and the fresh testicular virus were freed from cells. Apparently all cells were not removed from the serum, because they state that in hanging drop preparations of the medium "occasional red blood cells from the rabbit serum" were seen. Furthermore, the amounts of the different ingredients in their medium are described in one ambiguous sentence: "Equal parts of fresh rabbit serum and Tyrode's solution were added to the kidney extract to make a dilution of 1 in 50 of the virus seeding." This sentence can be interpreted in several ways, but, in view of the work on the cultivation of vaccine virus that has been described by different investigators, we have chosen to interpret it in the following manner: 1 part of Tyrode's solution, 1 part of rabbit serum, and 1 part of kidney extract were mixed and to this mixture sufficient vaccine virus was added so that the dilution of the virus was 1 in 50.

Virus.—Two strains of vaccine virus were used in the experiments. One was a dermal strain (5) obtained from the New York City Board of Health, the other was the Levaditi neuro-vaccine virus. Both strains can be propagated without difficulty by passages through rabbit testicles or by cultivation in modified tissue cultures (5, 6) and are free from ordinary aerobic and anaerobic bacteria. When a virus was used to initiate a series of cultures, care was taken to remove or to kill all cells in the virus emulsion by thorough centrifugation followed by repeated (3 times) freezing and thawing of the supernatant virus-containing material. CO_2 snow ($-78^\circ\text{C}.$) was used for freezing.

Tyrode's Solution.—Tyrode's solution prepared according to the following formula was sterilized by filtration: NaCl , 8.0 gm.; KCl , 0.2 gm.; CaCl_2 , 0.2 gm.; MgCl_2 , 0.1 gm.; NaH_2PO_4 , 0.05 gm.; NaHCO_3 , 1.0 gm.; glucose, 1.0 gm.; water, q.s., 1,000 cc.

Serum.—Serum obtained from defibrinated rabbit blood was used in accordance with Eagles and Kordi's instructions. We were careful, however, to remove as many cells as possible by centrifuging it twice, 1 hour on each occasion, at approximately 3,600 R.P.M.

Tissues.—In our experiments, media containing viable cells were used as controls. Testicular and renal tissues were obtained from young rabbits (half-grown). Chick embryo tissue was obtained from eggs incubated 11 or 12 days. Inasmuch as only small quantities of tissue were required for the controls in each experiment, the remainder of the kidneys, testicles, or chick embryos was used for the preparation of tissue extracts.

when the wash fluid no longer contained egg albumin, as determined by the addition of precipitin, the coated particles were resuspended in sodium chloride. This suspension served as the antigen and portions of it were tested with anti-egg albumin serum. After the addition of immune serum the tubes were incubated for 2 hours and centrifuged 45 minutes at 2500 R.P.M. The effect on volume is recorded in Table I. In this experiment an International Equipment Company, Size 1 centrifuge was employed.

In such experiments it is not necessary to take precautions to insure stability of the suspensions of collodion particles.

There are indications that collodion particles behave much the same as specifically agglutinated bacteria, provided they have been sensi-

TABLE I
Volumetric Change in Collodion Particles Sensitized with Egg Albumin and Subsequently Tested with Egg Albumin Precipitin

Dilution of serum	Volume	Agglutination
	<i>c.mm.</i>	
1:10	6.50	Zone of rapid flocculation
1:20	6.50	
1:40	6.50	
1:80	5.80	
1:160	5.63	
1:320	5.28	
1:640	5.10	Slow precipitation
Control	5.10	

tized with antigen and then treated with immune serum. It should be stated, however, that in experiments of this type the centrifugation was sufficient to clear the supernatant but not to insure complete packing.

The use of collodion particles seemed to offer an approach to the question of the relation of the volume increase to actual protein absorption. The sensitized collodion particles give no color when phenol indicator is added and additions to them of protein from the immune serum should be measurable.

Experiment 2 correlates volumetric increase with protein absorption by means of sensitized collodion particles specifically agglutinated.

bits of ground tissue, (3) bits of ground tissue treated 1 hour at room temperature with hypertonic salt solution, (4) bits of ground tissue treated with hypertonic salt solution and then frozen and thawed once, suspended in mixtures of serum (1 part), Tyrode's solution (2 parts), and vaccine virus; (5) supernatant fluids (1 part), obtained after the first centrifugation of the extracts described above, mixed with rabbit serum (1 part), Tyrode's solution (1 part), and vaccine virus. These controls were handled, titered, incubated, and again titered in a manner similar to that used for cultures set up with the supernatant fluids obtained after the second centrifugation of the tissue extracts.

In certain instances, in addition to the controls already enumerated, plasma-tissue preparations of the minced tissues, the ground tissues, the tissues treated with hypertonic salt solution at room temperature, and the tissues treated with hypertonic salt solution and then frozen and thawed once were also made to determine if the treatment with the salt solution followed by a single freezing and thawing really killed the cells. These controls were made when the extracts were prepared from rabbit testicles and chick embryos because such tissues are highly suitable for cultivation in plasma. The usual cover-slip method was employed in which bits of tissue were embedded in thoroughly centrifuged rabbit plasma undiluted or diluted with an equal amount of Tyrode's solution and clotted with chick embryo extract that had been freed from viable cells by means of centrifugation and repeated freezing (3 times with CO₂ snow) and thawing. The cultures were incubated at 37°C. for a week and were examined daily for evidences of growth of cells.

Sterility of Cultures.—Each culture was tested for the presence of ordinary aerobic and anaerobic bacteria, and all cultures contaminated with such organisms were discarded.

Rabbit Kidney Extracts

Three attempts to cultivate a dermal strain of vaccine virus in kidney extracts prepared in the manner described above were unsuccessful. The results of one experiment are summarized in Table I and show that the virus did not grow in the extracts nor in the presence of viable kidney tissue suspended in a mixture of serum (1 part) and Tyrode's solution (2 parts), but did increase in amount in the medium composed of bits of testicular tissue suspended in a mixture of kidney extracts (1 part), serum (1 part), and Tyrode's solution (1 part). One attempt to cultivate the Levaditi neuro-vaccine virus in kidney extracts was also unsuccessful. The results of this experiment are summarized in Table II and reveal that the virus multiplied abundantly in the extract media if bits of viable testicular tissue were added, but failed to increase in amount in any of the other media.

absorb from the immune serum nearly four times as much protein as is indicated by change in volume.

These unexpected findings will be considered in the discussion. The experiments, as such, indicated that the degree of protein adsorption from the immune serum could be measured by a colorimetric method. It seemed probable that the physical character of the collodion particles was not identical with that of microorganisms and that the results might be influenced by differences in contour. Hence it was determined to measure the quantity of protein absorbed by bacteria from specific agglutinating serum and at the same time to measure changes in volume.

The details of two experiments follow.

Experiment 3.—A similar procedure was employed in two experiments, one with *B. abortus*, the other with *B. aertrycke*, and their respective sera. Antigens were prepared from young agar cultures suspended in an excess of salt solution and centrifuged, and the organisms were resuspended in fresh salt solution. These antigens were distributed in 250 cc. centrifuge bottles in quantities of 100 cc. To half the bottles 100 cc. of diluted immune serum was added and to the remainder a like amount of sodium chloride solution. The mixtures were incubated 2 hours, refrigerated 4 hours, centrifuged at 3000 R.P.M. for 1½ hours, and refrigerated overnight. The supernatant was poured off, fresh salt solution added, and the centrifugation repeated. After three washings all the supernatant was withdrawn and 2 cc. of normal sodium hydroxide was added to the sediment and later 23 cc. of a 50 per cent, by weight, solution of urea. After warming to 40°C. the mixture became sufficiently clear for colorimetric determinations. The quantity of protein was determined in the unit of antigen and in a similar unit which had been treated with agglutinating serum.

Changes in volume were determined by means of the capillary centrifuge tubes. These changes in volume together with the approximate size and numbers of the organisms are given in Table III.

The data in Table III show that the increase in volume cannot be attributed entirely to protein deposition from the immune serum during agglutination. In the instance of *B. abortus*, the increase in volume approximated six times that of the quantity of protein taken up by the bacilli. In the case of *B. aertrycke* the ratio of volume increase to protein increase was $2\frac{1}{2}$ to 1. It is clear then that only a fraction of the change in cubic content can be attributed to actual protein deposited on the surface of the organism. The experiments are of in-

Rabbit Testicle Extracts

One attempt to cultivate a dermal strain of vaccine virus in rabbit testicular extracts was unsuccessful. The results of the experiment, summarized in Table III, show that the virus multiplied in media containing minced and ground tissues, but failed to grow in other kinds of media. Plasma cultures of the minced tissue, the ground tissue,

TABLE III

Cultivation of Vaccine Virus in Testicular Extracts

Time of titration	Minced testicle tissue	Ground testicle cells	Testicle cells treated with salt at room temperature	Testicle cells treated with salt and frozen	1st supernatant from medium prepared at room temperature	1st supernatant from medium prepared by freezing	2nd supernatant from medium prepared at room temperature
Before incubation	1,000	1,000	1,000	1,000	1,000	1,000	1,000
After 4 days incubation. 3 flasks pooled	1,000,000	10,000	Undiluted	1,000	100	10	10
Percentage of plasma preparations showing growth of cells	100	37.5	12.5	5.5			

Summary of results obtained in an attempt to cultivate a dermal strain of vaccine virus in extracts of testicular tissue. The strain of virus has been propagated by serial testicular passages in rabbits and a testicular emulsion containing the virus was used to inoculate the cultures. The cultures contained the material listed at the head of each column plus serum and Tyrode's solution. The virus multiplied in the media containing minced testicle and ground testicle. The multiplication, however, was distinctly less marked in the latter. No multiplication occurred in any of the other media. It will be noted that grinding injured the cells considerably but that treatment with salt and freezing (once) did not kill all of the cells.

and the tissues treated with hypertonic salt solution and freezing and thawing once were made. The percentage of slides that showed growth of cells is also recorded in Table III and indicates that all of the cells in testicular tissue were not killed by the method employed by Eagles and Kordi (4) in the preparation of their extracts. An attempt to cultivate the Levaditi neuro-vaccine virus in testicular extracts was

granular organisms, difficult to suspend in sodium chloride, are employed as antigens. In such instances although all the organisms flocculate spontaneously, the differences in antigenic volume between the tubes containing immune serum and those without serum argue that antibody has combined with the bacterial cell.

The experiments are of interest from a more general standpoint. We have shown that the increase in volume is not directly due to the deposition of protein from the immune serum. Collodion particles take up much more protein than is indicated by change in volume, whereas bacteria take up less. Calculations show that if the protein taken up by *B. abortus* were spread over the whole cell surface, the layer would be about 0.025μ thick, whereas *B. aertrycke*, under the conditions of Experiment 3, would be covered by a layer of 0.05μ thick. Neither quantity could increase the volume of the organism to the degree of the actual findings.

It is possible to reconcile the opposed findings with bacteria and collodion by assuming that the surfaces of collodion particles are much rougher than those of bacteria. In all probability much of the antigen lodges in crevices or pores in the collodion and when the antibody protein is deposited on exposed antigen it fails to increase the particle surface appreciably. On the other hand, bacterial surfaces must be regarded as relatively smooth and the quantity of serum protein deposited appears to be proportional to the quantity of antigen and concentration of the antibody. The collodion particle probably possesses a surface out of all proportion to its diameter, while the bacteria's surface is directly proportional to its diameter. Such reasoning seems to us to explain the quantitative difference in the amount of serum protein absorbed by the sensitized collodion particle and by a micro-organism under essentially the same conditions.

It remains to advance an explanation which could account for volumetric differences of the agglutinated and unagglutinated bacteria. It has been shown that the actual protein absorbed is insufficient to account for the difference. Should the protein be in the form of a thin layer, four to eight molecules thick² over the whole surface, then, when the organisms are packed by prolonged centrifugation, the

² These calculations were made from the data in Table III and the diameter of a molecule considered as 6.2×10^{-6} mm.

Figs. 1 and 2 are photographs of the growth of cells that had been treated in such a manner.

Two attempts to cultivate the Levaditi neuro-vaccine virus in extracts of chick embryos were unsuccessful. In each instance, a fresh testicular virus emulsion was used to initiate the cultures and one set of subcultures was made. In addition to the control cultures usually prepared, one was made with cells frozen (CO₂ snow) and thawed 5

TABLE V
Cultivation of Vaccine Virus in Chick Embryo Extracts

Time of titration	Minced embryo tissue	Ground embryo tissue	Cells salted at room temperature	Cells salted and frozen	1st supernatant from cells salted at room temperature	1st supernatant from cells salted and frozen	2nd supernatant from cells salted at room temperature	2nd supernatant from cells salted and frozen
Before incubation	100,000	100,000	100,000	100,000	100,000	100,000	100,000	100,000
1st culture. 3 flasks pooled	100,000	100,000	> 100,000	> 100,000	1,000	100	1,000	100
2nd culture. 3 flasks pooled	1,000,000	1,000,000	1,000,000	100	—	—	—	Undiluted
Percentage of plasma preparations showing growth of cells	66	100	72.5	81.2	—	—	—	—

Summary of results obtained in an attempt to cultivate a dermal strain of vaccine virus in extracts from chick embryos. The different media were made as described in the text and the cultures contained the material listed at the head of each column plus serum and Tyrode's solution. Multiplication of the virus occurred in media containing viable cells; none occurred in any of the other media. The plasma preparations showed that treatment of chick embryo cells with salt and a single freezing did not kill all of the cells. At the time transfers were made, the virus underwent a twenty-fivefold dilution. The first set of cultures was incubated 3 days; second set, 4 days.

— indicates that no virus was demonstrable.

times. Plasma cultures of the minced tissue, the ground tissue, the tissue treated with hypertonic salt solution and freezing (ice box) and thawing once, and the tissue frozen (CO₂ snow) and thawed 5 times were also set up. The results obtained in one of the experiments are summarized in Table VI and show (1) that the virus multiplied in 2 successive cultures only in the medium containing bits of minced tissue; (2) that growth of cells was found in plasma preparations of

all the bacteria were agglutinated. We have attempted to correlate the volumetric increase with the quantity of protein adsorbed by the organisms during agglutination and have studied not only bacteria but collodion particles first sensitized to antigen and then agglutinated with a precipitin specific for the antigen. The increase in volume of the collodion particles was small and the quantity of protein adsorbed relatively large. When two species of bacteria were agglutinated with their respective antisera the reverse was true; the apparent volume increase was much greater than the quantity of protein deposited during the reaction. There is, then, no direct correlation between protein deposition and apparent increase in volume. Nevertheless, the results of experiments here reported have suggested an explanation for the fact of increase in volume.

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Furthermore, treatment of rabbit testicular tissue and chick embryo tissue in the manner described by Eagles and Kordi for the preparation of the extracts leaves some cells not only alive but capable of proliferation. Although the results of our work are not in accord with those obtained by Eagles and Kordi, we offer no explanation for the discrepancy. Nevertheless, one cannot examine the results of our work recorded in the six tables without recognizing the fact that in the types of media used the presence of viable cells appears to be essential for the multiplication of vaccine virus. Rabbit testicular tissue and bits of chick embryos support the regeneration of the active agent more efficiently than does rabbit renal tissue.

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EXPLANATION OF PLATE 45

FIG. 1. Photograph of a tissue culture prepared from chick embryo tissue that had been treated with hypertonic salt solution for 1 hour at room temperature. Note the evidence of active growth of cells. Unstained. $\times 30$.

FIG. 2. Photograph of a tissue culture prepared from chick embryo tissue that had been treated with hypertonic salt solution followed by freezing (ice box) and thawing once. Note the evidence of active growth of cells. Unstained. $\times 30$.

FURTHER OBSERVATIONS ON THE CULTIVATION OF VACCINE VIRUS IN LIFELESS MEDIA

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PLATE 45

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In a previous paper (1) we reported the results of attempts to cultivate vaccine virus in a "cell-free" medium described by Eagles and McClean (2, 3). We were unable to confirm the findings of these workers. Following the presentation of our paper for publication, a report by Eagles and Kordi (4) appeared in which they stated that they also were able to cultivate the virus in the absence of cells. Inasmuch as these investigators used a medium somewhat different from that employed by Eagles and McClean, it seemed advisable for us to determine whether we were able to confirm their findings. This paper records the results of our work dealing with the matter.

EXPERIMENTAL

Eagles and Kordi (4) stated that they were able to obtain growth of vaccine virus in a medium consisting of a mixture of Tyrode's solution, rabbit serum, and an extract prepared from rabbit kidney tissue by means of a hypertonic salt solution and freezing. We have repeated their experiments according to the directions given in their paper. In view of the fact that vaccine virus multiplies more consistently in media containing bits of rabbit testicles or chick embryos than it does in media containing minced rabbit kidney (1), it seemed expedient to supplement Eagles and Kordi's experiments by the use of extracts of these tissues. Furthermore, it seemed advisable to use at least two strains of vaccine virus.

We say that we repeated Eagles and Kordi's (4) experiments according to the directions given in their paper. We realize, however, that we may not have accomplished this, because these workers, as did

Extracts of Renal Tissue.—Extracts of renal tissue were made in the following manner: Both kidneys from a half-grown rabbit were removed aseptically. After the fat and pelvic tissue had been removed, the remaining kidney tissue, which weighed approximately 10 or 11 gm., was divided into equal parts, placed in mortars, and thoroughly ground without the addition of an abrasive. Then each portion was mixed with 1 cc. of a 9 per cent NaCl solution. One portion was allowed to stand 1 hour at room temperature after which time 9 cc. of sterile distilled water were added. The other portion was placed in the freezing compartment of a frigidaire (approximately $-10^{\circ}\text{C}.$) where it was allowed to remain for 1 hour, during which time it became frozen. After it had been removed from the ice box and rapidly thawed, 9 cc. of sterile distilled water were added. Each portion was then placed in a tube and centrifuged for 1 hour at a speed of approximately 3,600 R.P.M. The supernatant fluids were removed and again centrifuged for an hour at high speed. The supernatant fluids after the second centrifugation constituted the kidney extracts used in the preparation of the media. In each instance the supernatant fluids were carefully removed by means of 10 cc. pipettes, the tips of which were made of medium size capillary tubing. Such pipettes were used in order to avoid the fat floating on the surface of the fluids as well as the sedimented cells.

Extracts of Testicular Tissue and Chick Embryo Tissue.—Testicular extracts and extracts of chick embryo tissue were prepared in a manner similar to that used for the preparation of renal extracts with the exception that 4 rabbit testicles (approximate weight 7-9 gm.) or 4 chick embryos (11 days old and weighing approximately 8-9 gm.) were used instead of 2 kidneys.

Preparation of Cultures.—Inasmuch as two kinds of extracts were made from the tissues, e.g. one made at room temperature, the other obtained by freezing and thawing, two sets of cultures were prepared in each experiment. The cultures were prepared in the following manner: 1 part of extract centrifuged twice, 1 part of serum centrifuged twice, and 1 part of Tyrode's solution were mixed and seeded with vaccine virus (the amount used was sufficient to insure the presence of considerable virus) from which viable tissue cells had been removed or killed by centrifugation and repeated freezing and thawing. The material was then distributed in 2 cc. amounts to Carrel D flasks. Usually there were 3 flasks for each kind of medium.

Incubation of Cultures.—The cultures were incubated at $37^{\circ}\text{C}.$ for 3 or 4 days.

Titration of Virus in Cultures.—The titer of the virus in the cultures before incubation was determined by means of intradermal inoculations (0.2 cc.) in rabbits of dilutions of the cultures. After incubation the titer was again determined in the same manner. At first the material in each flask was titered. It was found, however, that such a procedure was not essential for this work and then all the flasks (usually 3) containing similar cultures were pooled before titration.

Controls.—Each time extract cultures were prepared, a number of control cultures were made and usually consisted of (1) bits of minced viable tissue, (2)

tions of high tinctorial value resulted in an immediate entrance of the coloring matter into the minute lymphatics of the locality rendering them brilliantly visible. The anatomy of the lymphatics and the secondary distribution of the dyes could be readily followed. The permeability of the wall of the lymphatic capillaries could be gauged by observing under the microscope the rate of escape of dye from the intact channels into which it had passed.

Several dyes were used, in special the highly diffusible patent blue V and Neptune blue, and the very poorly diffusible pontamine sky blue and Chicago blue (1, 6-8). Because of the tendency of these latter to leave a long-enduring mark, owing doubtless to storage in the local macrophages, most of the experiments were done with readily diffusible dyes, which are only temporarily stored if at all (9, 10). Save in a few exceptional instances noted as such, the dyes were used in aqueous solution isotonic with 0.9 per cent sodium chloride, as determined by freezing point determinations with a Beckman apparatus. Of the materials employed, pontamine sky blue¹ is isotonic with blood in a 21.6 per cent watery solution, Chicago blue² in 17.1 per cent, patent blue V² in 11 per cent and Neptune blue² in 5.5 per cent (1, 3). The amount of dye present in such percentages was far greater than was necessary for the work. Consequently the autoclaved solutions were diluted with Tyrode's solution sterilized by filtration at the time when the experiment was to be done. Sterile homologous serum obtained by vein puncture was occasionally used as a vehicle for the dye.

For the injection, a uniform technic was employed. The freshly prepared solution of dye was taken into a syringe graduated to 0.01 cc. both barrel and plunger, and prepared by dry sterilization. The skin of the volar surface of the forearm, or unfrequently of the inner surface of the lower thigh was gently cleansed with tincture of green soap and sponged off with 60 per cent alcohol, with avoidance of rubbing. The region for injection was then covered with a dry, sterile sponge, the syringe was capped with a 29 gauge hypodermic needle or a special 30 gauge platinum iridium needle; with the free hand the region to be injected was firmly grasped from beneath and the skin was gently drawn taut. The injecting needle was now thrust superficially into the epidermis at an angle of 45° and with the bevel down, was brought as nearly parallel with the skin surface as possible and inserted further. Rocking the bevel of the needle a trifle, and at the same time expressing a minute quantity of dye, caused the torn lymphatic capillaries to take up the colored material immediately. Even the gentlest pressure frequently caused some of the dye to flow backwards about the shaft of the needle and escape to the skin surface, obscuring the injection field and marring the test.

Most of the injections were made under the dissecting microscope. To increase visibility sterile neutral paraffin oil was flooded on the skin either before or after insertion of the needle.

¹ Du Pont Dyestuffs Corporation.

² General Dyestuffs Corporation.

TABLE I
Cultivation of Vaccine Virus in Kidney Extracts

Time of titration	Mixed kidney extracts plus testicular cells, serum, and Tyrode's solution	Serum, Tyrode's solution, and kidney cells	Kidney extract prepared at room temperature plus serum and Tyrode's solution	Kidney extract prepared by freezing plus serum and Tyrode's solution
Before incubation	20,000	20,000	20,000	20,000
After incubation for 4 days	2 flasks pooled 100,000	3 flasks pooled 1,000	Flask 1 100 " 2 100 " 3 100	Flask 1 100 " 2 100 " 3 100

Summary of results obtained in an attempt to cultivate a dermal strain of vaccine virus in extracts of rabbit kidney. The strain of virus employed has been cultivated for several years in modified tissue cultures. The virus multiplied in the medium containing testicular cells but decreased in amount in the extract media and in the medium containing kidney cells. The titer of the virus varied very little, if any, in the different flasks containing extract media.

TABLE II
Cultivation of Vaccine Virus in Kidney Extracts

Time of titration	Mixed kidney extracts plus minced testicular tissue	Minced kidney tissue	Ground kidney tissue	Kidney tissue salted at room temperature	Kidney tissue salted and frozen	1st supernatant from kidney tissue salted at room temperature	1st supernatant from kidney tissue salted and frozen	2nd supernatant from kidney tissue salted at room temperature	2nd supernatant from kidney tissue salted and frozen
Before incubation	10,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000
After 4 days incubation. 3 flasks pooled	1,000,000	10,000	1,000	10	10	100	1,000	100	100

Summary of results obtained in an attempt to cultivate the Levaditi neuro-vaccine virus in extracts from rabbit kidney tissue. The different media were made as described in the text and the cultures contained the material listed at the head of each column plus serum and Tyrode's solution. Multiplication of the virus occurred in the extract medium containing viable testicular cells; the virus maintained its original titer in the medium containing minced kidney tissue; the titer decreased in all other media.

the last one shown in Fig. 1. Escape took place later from the other dye-containing channels.

It is plain that the lymphatic capillary wall is highly permeable for the very diffusible patent blue V. So rapid and abundant was the escape in such an experiment as that just described, that within a few minutes the definition of the individual capillaries was lost save at the margin of the stained region. By this time, too, the tissue into which the needle had been thrust had become distinctly edematous. Within an hour, the edema had spread and with it the diffuse interstitial coloration as well. For the next few hours, secondary dispersion of the dye continued while the color intensity of the stained region faded proportionally. After 8 to 10 hours it became possible once again to see the minute blood vessels around the site of puncture, and edema and hyperemia were but barely perceptible. There was no pain or tenderness. By the next day, the coloration of the skin had disappeared, leaving only faint traces which remained for 48 or even 72 hours. The site of the needle puncture remained visible sometimes as a colored point for several days, but the regions that dye had reached only by secondary escape from injected lymphatics were always practically decolorized within 24 hours. The urine shows traces of the dye as early as 1 hour after the injection and occasionally for as long as 18 hours.

When 0.1 cc. of the dye was very rapidly injected there resulted a localized, sharply demarcated swelling elevated 1 to 3 mm., which spread to a diameter of only 1 to 3 cm., instead of the greater distance observed when the injection had been slow. The removal of dye was more rapid than in experiments such as that just described.

The Permeability of the Lymphatic Capillaries in Human Skin

In earlier work from this laboratory the rate of the escape of vital dyes of known diffusibility from lymphatic capillaries of the mouse ear has served as a means of studying the permeability of their walls and the factors influencing this permeability. Our present experiments have shown that pontamine sky blue escapes more slowly from the intact lymphatic capillaries of normal human skin than does Chicago blue in equimolecular solution, while patent blue V, and also Neptune blue, escape more readily than either of the pigments first mentioned, the rate of escape of all four dyes being roughly proportional to their diffusibility through collodion membranes. This is true also of the entrance of these dyes through the intact lymphatic wall, as will be brought out further on in the paper. Increasing the concentration of any one of the dyes quickens its rate of escape while a decrease retards it. The presence of human blood proteins (serum admixture)

also unsuccessful. In this experiment one set of subcultures was made and the results of the work recorded in Table IV show that the virus multiplied only in the media containing minced testicular tissue.

Chick Embryo Extracts

One attempt to cultivate a dermal strain of vaccine virus in extracts of chick embryos was unsuccessful. In this experiment a set of sub-

TABLE IV
Cultivation of Vaccine Virus in Testicular Extracts

Time of titration	Minced testicular tissue	Ground testicular tissue	Testicular tissue salted at room temperature	Testicular tissue salted and frozen	1st supernatant from testicular tissue salted at room temperature	1st supernatant from salted and frozen testicular tissue	2nd supernatant from testicular tissue salted at room temperature	2nd supernatant from salted and frozen testicular tissue
Before incubation	100,000	100,000	100,000	100,000	100,000	100,000	100,000	100,000
1st culture. 3 flasks pooled	10,000,000	100	100,000	100	10,000	10,000	10,000	10,000
2nd culture. 3 flasks pooled	10,000,000	Undiluted	10	—	—	—	—	—

Summary of results obtained in an attempt to cultivate the Levaditi neurovaccine virus in extracts from rabbit testicular tissue. The different media were made as described in the text and the cultures contained the material listed at the head of each column plus serum and Tyrode's solution. Each set of cultures was incubated 4 days. Multiplication of the virus occurred only in the medium containing minced testicular tissue.

At the time transfers were made, the virus underwent a twenty-fivefold dilution. — indicates that no virus was demonstrable.

cultures was also made. Plasma cultures of the minced tissue, the ground tissue, and the tissues treated with hypertonic salt solution and a single freezing and thawing were set up. The results obtained are recorded in Table V and reveal (1) that the virus multiplied only in the media containing bits of minced tissue, ground tissue, or tissue treated with hypertonic salt solution for 1 hour at room temperature; (2) that all chick embryo cells were not killed by grinding and treatment with hypertonic salt solution and freezing and thawing once.

capillaries frequently failed to be entered by it. Many injections were made in each specimen, using for some the same technic which was employed to fill the lymphatics with dye during life. In other instances, to prepare a way for the injection, a finely ground dissecting needle was thrust into the corium and then pushed parallel to the skin surface for 2 to 4 cm. The procedure carried out under the binocular microscope, ruptured many of the superficial lymphatic capillaries, and when a 30 gauge platinum hypodermic needle was forced into the puncture track and injecting fluid introduced through it, many more lymphatics of the superficial plexus filled, at times over a larger area, than in the ordinary test in the living subject. Both methods of injection of the skin specimens gave similar findings, save that the one just described yielded more completely injected preparations.

At once after injection all specimens were chilled in normal saline solution and fixed in acid-formalin or alcohol. For examination some of the injected regions of each specimen were sectioned in the usual manner, others were cleared and preserved in oil of wintergreen for study, and still others, which had been cleared, were stained in solid slices about 1 mm. thick with dilute hematoxylin and eosin, yielding most beautiful preparations which showed clearly the position and relationships of the injected superficial lymphatic capillaries.

All specimens regardless of the age or sex of the individual, yielded pictures of the lymphatics having similarities either to those of Figs. 1 or 2, every gradation between the two being found. In certain specimens a rich superficial plexus of lymphatics was readily disclosed while in other specimens fewer of the superficial lymphatics and more of the deeper draining trunks took up the injection fluid. But even in the latter specimens, it was possible to show the presence of the rich superficial plexus—if several injections were made with great care under the microscope. Because of individual peculiarities of the skin the needle tended, in some specimens, to pass too deeply or else not deeply enough to inject the superficial plexus well. To this simple cause the pronounced difference in results seemed wholly attributable. More will be said of the results in a subsequent paper.

Cleared and stained, injected specimens and sections thereof show the lymphatics described by others, namely superficial lymphatic capillaries lying in the papillary stratum of the corium with blind ends extending into the papillae—and a deeper capillary plexus in the lower layer of the corium and the tela subcutanea. The existence of these plexuses has long been known and recently their relationships have been well shown by diagrams such, for example, as that of Neumann (10) reproduced from von Brunn (11) in Bartels' monograph (12). In

minced tissue, ground tissue, and tissue treated with hypertonic salt solution and freezing (ice box) and thawing once, while no growth was observed in preparations of cells frozen (CO₂ snow) and thawed 5 times.

TABLE VI
Cultivation of Vaccine Virus in Chick Embryo Extracts

Time of titration	Minced embryo tissue	Ground embryo tissue	Cells salted at room temperature	Cells salted and frozen in ice box	Cells salted and frozen with CO ₂ snow	1st supernatant from cells salted at room temperature	1st supernatant from cells salted and frozen in ice box	2nd supernatant from cells salted at room temperature	2nd supernatant from cells salted and frozen in ice box
Before incubation	10,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000	10,000
1st culture. 3 flasks pooled	>1,000,000	>1,000,000	>1,000,000	100	100	1,000	1,000	100	1,000
2nd culture. 3 flasks pooled	1,000,000	100	100	—	—	—	—	10	—
Percentage of plasma preparations showing growth of cells	100	83	78	67	0				

Summary of results obtained in an attempt to cultivate the Levaditi neuro-vaccine virus in extracts from chick embryos. The different media were made as described in the text and the cultures contained the material listed at the head of each column plus serum and Tyrode's solution. One set of subcultures was made. Multiplication of the virus in 2 successive sets of cultures took place only in the medium containing minced chick embryo tissue. The plasma preparations showed that treatment of chick embryo cells with salt and freezing (once at -10°C.) did not kill all the cells, while no growth of cells was obtained from tissue that had been frozen (CO₂ snow) and thawed 5 times.

At the time transfers were made, the virus underwent a twenty-fivefold dilution.

The first set of cultures was incubated 4 days; the second set, 3 days.

— indicates that no virus was demonstrable.

SUMMARY AND CONCLUSIONS

We have made ten attempts to cultivate vaccine virus in tissue extracts prepared according to the method described by Eagles and Kordi (4). Renal, testicular, and chick embryo extracts were employed with a dermal strain of vaccine virus and with the Levaditi strain of neuro-vaccine virus. In no instance were we able to show that the virus multiplied in the extract media. Both of these strains of virus, however, multiplied in media containing bits of minced viable tissue.

described although the latter author had written much upon the theme of skin lymphatics (17). We find anastomoses to be abundant. Doubtless they escaped Handley because of incomplete injection of the vessels.

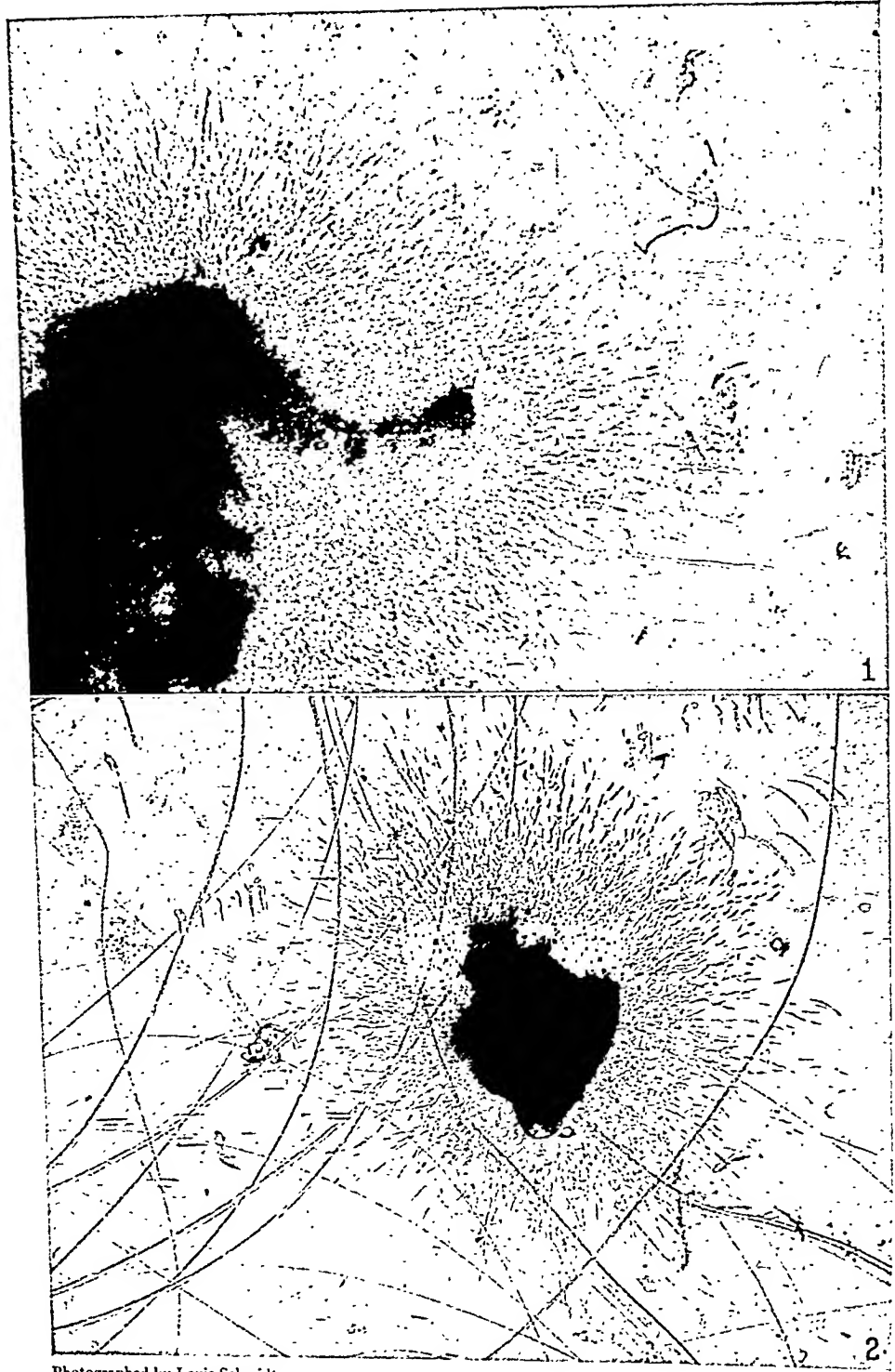
Many of the lymphatics extend at an inclined angle to the skin surface, communicating between the superficial and deep plexuses, and branching as they run. They do not directly link one capillary plexus with the other but form an almost continuous network. When a lymphatic injection of dye solution or ink suspension is successfully made the superficial plexus conducts the material for a distance of 3 to 5 cm. from the point of injection. If another successful injection is made 8 cm. from the first, the lymphatic capillaries carry material from the latter directly into some of the channels first injected, showing how direct are the anastomoses of these vessels. When four or five injections are spaced in the form of a circle, at a distance of about 5 cm. from each other, the superficial lymphatics of most of the enclosed area may become injected, once again showing the rich anastomosis.

The Lymphatic Involvement in Skin Injury

Invariably in our experience, colored substances injected intradermally on the surface of the forearm have entered the lymphatics directly. This fact prompted us to a further study to determine how considerable an injury is required for direct entrance into the skin lymphatics.

On the surface of the forearm, cleansed with soap and alcohol, linear scarifications about 1/2 cm. long and 2 cm. apart were made, single scratches with a sharp, sterile needle manipulated under the binocular dissecting microscope. Only the superficial epithelium was removed, no skin venules or capillary tufts were visibly torn, and there was no bleeding. To facilitate consecutive observations after known periods of time the scarifications were made at 2 minute intervals. Equimolecular isotonic solutions of pontamine sky blue, Chicago blue and patent blue V were dropped upon the scarifications and immediately covered with sterile neutral paraffin oil to prevent drying.

As one watched, the dye appeared in numerous lymph capillaries leading away from the lesions, these becoming visible under the microscope in 1 to 6 minutes because of their colored contents. In later experiments, we found it best to allow an interval of 6 to 8 minutes between the scarification and the dropping on of the dye, else some irregularity in the findings would occur as the result of wheal formation at the margins of scratch. Work to be reported below has shown that during the formation of histamine wheals, the lymph vessels undergo compression by the edema of the wheal. After the interval mentioned, patent blue V applied to the



Photographed by Louis Schmidt

(Rivers and Ward: Vaccine virus in lifeless media)

est penetration beneath the epithelium opens these vessels to the entrance of particulate matter.

The Rate of Lymph Transport

When as little as 0.05 to 0.10 cc. of isotonic dye solution is slowly injected into normal skin the phenomena resemble those already described. Dye spreads within the lymphatic plexus over an area 2 to 3 cm. in diameter, and within 3 to 4 minutes some of the dye escapes secondarily into the interstitial tissue through the walls of the capillaries containing it. The remainder is seen extending somewhat diluted into lymph channels not filled originally, notably into the larger lymphatics draining the region. 5 minutes after the injection dye can usually be seen 15 cm. away from the injection spot in the draining lymphatic trunks, rendering visible those which lie in the subcutaneous fat. In two instances dye has been perceptible in the draining trunks of the axillary portion of the arm 8 minutes after an intradermal injection of 0.10 cc. of patent blue V into the volar surface of the forearm.

The arm in these experiments had throughout rested upon a table, with the elbow slightly flexed, and the shoulder dropped so that the entire limb lay at the heart level. In ten other instances, after similar injections, the dye appeared in the axillary trunks within 10 to 18 minutes. During the period of observation these small injections of isotonic dye solution gave rise to local swelling which required 5 to 10 minutes to form. In estimating from these experiments the rate of lymph transport in untouched skin the factor of pressure at the injection site cannot be ruled out. However, the fact that the swelling required several minutes to form and that before this happened, dye had passed in draining trunks several centimeters distant from it, suggests that the escape of fluid from the blood as result of the puncture injury had only a slight degree of influence, if any, upon the experimental results.

In Fig. 7 is pictured an instance of rapid lymph drainage. The photograph was taken 6 minutes after two simultaneous injections of 0.02 cc. of patent blue V into the individual M. The black, oblong mark on the skin surface near the site of the upper injection is due to dye that passed back along the needle track. The net-like appearance of the injected lymphatic plexus is not seen because already the secondary escape of dye through the walls of these vessels has obscured it, with result a deep and even color of the originally injected region. Immediately surrounding this latter a few lymph capillaries have been rendered visible by dye content and stand out as twig-like extensions. The draining trunks extending up the arm are seen in a lighter shade of the dye. They could be followed almost to the axilla with the unaided eye, a fact which does not appear in the photograph.

THE LYMPHATIC PARTICIPATION IN HUMAN CUTANEOUS PHENOMENA

A STUDY OF THE MINUTE LYMPHATICS OF THE LIVING SKIN

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PLATES 46 TO 49

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It is common knowledge that human skin is supplied with a close network of minute lymphatics; yet the physiology of these channels remains obscure, little being known of what takes place in them under normal conditions, and still less of what goes on during inflammation and under other circumstances of injury. The lymphatics of the skin are so constituted that obviously they must be concerned in fluid exchange and fluid transport. Yet because little is known of their functionings they have been generally ignored in appraising skin phenomena.

To determine the share of the lymphatics in skin phenomena it is essential to develop a method whereby these vessels can be studied in the living tissue. Recently we have found (1-5) that the injection of vital dyes into the superficial layer of the skin of mice, rats and rabbits renders the lymph channels plainly visible in regions accessible to study, for example the ear. It has been noted that the walls of the vessels respond promptly to various stimuli, often with marked changes in their permeability. The method has now been adapted to human skin, disclosing facts that form the subject of the present paper.

Procedures

For the greater part of the work the authors served as experimental material, submitting themselves to a series of intradermal and subcutaneous injections with vital dyes. The injections were made at intervals over a period of several months, care being taken in the later tests not to utilize skin areas which might conceivably have undergone changes as result of earlier ones. Supplementary experiments were carried out on six other volunteers. The injections with isotonic dye solu-

sive tape to the volar surfaces of the forearms of two individuals. The arms,—which had not been exposed to sunlight for several months—were subjected to irradiation for 3 minutes at 18 inches from a mercury vapor quartz tube ultraviolet lamp. The rectangular spots of unprotected skin developed a mild hyperemia in 3 hours, and by the next day, stood out upon a background of normal skin as brightly hyperemic and distinctly edematous inflamed areas. Injections of pontamine sky blue and patent blue V were made, within and just without these areas, as in the case of heated skin.

The lymphatics of the region of injury from ultraviolet light let dye through into the interstitial tissue far more readily than did those of normal skin and were the sooner obscured by it. The increased permeability of the lymphatic wall to patent blue V was so great in the injured region that the dye seemed to pass out at once through it, as if it offered practically no barrier at all. It escaped from the lymph capillaries so rapidly, that little or none passed directly to the draining vessels, but within 15 minutes it was visible in these as result of secondary resorption from the interstitial tissue.

Very striking were the results in three instances in which the dye injections were made at the margin of the irradiated area in such a way that about the same quantity of dye passed into the lymphatics of the inflamed area, and into the channels of the adjacent normal skin. For example, in one experiment, 5 minutes after the injection of 0.05 cc. of isotonic 11 per cent patent blue V, the hyperemic area receiving the dye showed a diffusely colored, deeply greenish blue spot in which no lymphatics whatever could be made out whereas in the adjacent normal skin, they could still be seen with some distinctness.

Effect of Local Injection of Toxins and Bacterins.—Two individuals, M and H, were tested for their response to intradermal inoculations of standard Schick test and Dick test toxins, to a concentrated streptococcus toxin,⁴ and to the standard typhoid vaccine of the New York Board of Health. As neither volunteer responded sharply to the Schick test material, it was not used further. The technic for the intradermal injection of toxins and vaccines was the same used for the dyes.

The concentrated streptococcus toxin caused within 20 hours after intradermal injection a development of large erythematous macules, slightly raised, moderately well defined and about 1.5 cm. in diameter. To cause this reaction 0.01 cc. suffices, the equivalent of one skin test dose. Scarlatinal toxin in skin test dose gave similar results, but the area of inflammation was about 2.5 cm. in diameter and the margins were not sharply defined.

Both subjects had received one or more courses of inoculation with typhoid

⁴ Kindly supplied to us by Dr. Homer Swift and Dr. Currier McEwen, of the Hospital of The Rockefeller Institute.

Demonstration of the Superficial Lymphatic Plexus

The sequence of events which follows an injection has been studied under the binocular microscope and photographed by a microcinema camera. Immediately upon introduction the dye solution passes into and along the lymphatic capillaries, rendering visible an exceedingly rich plexus of them which is all the more remarkable because it is wholly undisclosed by the ordinary means of examination of the skin (Figs. 1 to 6). As the injection continues, dye extends along the lymphatics (Figs. 1 and 2) often for a distance of 5 to 8 cm. from the needle point. This is best seen when slow, gentle pressure is exerted upon the injecting syringe, for under these circumstances the vessels take up most of the injected solution, only a little passing interstitially. When rapid injections are made, the lymphatic capillary plexus often becomes obscured by a localized intense coloration, only a few channels that drain from the margin of the colored area being visible (Fig. 7).

The photographs of Figs. 1 and 2 reproduce the findings at a magnification of approximately $3/2$. For purposes of detail Figs. 3, 4 and 5 give some enlargements of Figs. 1*a*, 1*d*, 1*e* respectively. In the test with the individual H (Fig. 1), 0.2 cc. of 11 per cent aqueous, isotonic solution of patent blue V was injected into the skin of the forearm. The dye was expressed from the syringe in about 80 to 85 seconds, only a little more slowly than in the ordinary clinical intradermal injection. The pictures selected represent the state of affairs at 15 or 30 second intervals, save for the last pictures of each figure which were taken 30 seconds after the termination of the injection. The pictures show the course of events during the injection and immediately after it.

Immediately that dye enters the tissue it passes directly into and along the lymphatic capillaries, even though the least possible pressure is put upon the syringe plunger. In the experiment of Fig. 1, within 30 seconds after beginning the injection the plexus of lymphatics showed dye over an area 14 to 15 mm. in diameter (Fig. 1*b*), and in 45 seconds the colored plexus was 2.5 cm. (Fig. 1*c*) wide. Within 1 minute and 15 seconds the outlines of the vessels first entered became blurred by dye that had escaped secondarily from them into the interstitial spaces through the lymphatic wall (Figs. 1*d* and 4). Thus the channels nearest the needle became completely obscured by a cloud of color. After the needle was withdrawn (Figs. 1*e* and 5), dye continued to extend along the channels at the periphery of the injected region. This extension after the injection is completed, and the abundant escape of dye from the channels in which it has been present longest is well seen in Fig. 6, an enlargement from a photograph taken about 5 minutes after

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in the injected solution acts to retard dye escape. The technic and the results of these experiments have been similar to those in our work upon the mouse (1) and hence need not be detailed here. But the existence of individual differences in the permeability of the skin lymphatics merits remark.

In repeated observations upon one of the two authors, isotonic patent blue V, 11 per cent in Tyrode's solution, passed through the lymphatic capillary walls in perceptible quantity within 1 to 2 minutes, whereas in the case of the other, 2 to 3 minutes was required. The difference could not be laid to variations in skin transparency since the lymphatics were so superficial that this could not enter as a factor. In five volunteers on whom we made a few tests each, the time of the first visible dye escape also differed, two showing some in 1 to 2 minutes and the other three in 2 to 5 minutes. The findings were consistent in repeated injections of any one subject. One cannot conclude, however, that individual differences exist in the intrinsic permeability of the wall of the lymph capillaries since many other factors can be invoked which will suffice to explain these differences.

Anatomy of the Cutaneous Lymphatics, with Reference to Individual Differences

Dye can be introduced into the lymphatic capillaries of some individuals with great ease, whereas into those of others only with difficulty, the injection material being almost at once led off into the deeper draining trunks. Figs. 1 and 2 serve excellently as examples of the major differences consistently found in experiments on individuals H and M as well as in five other volunteers.

To determine the cause for the individual differences, 24 skin specimens were injected and studied. The material, taken either at a prompt postmortem examination or at surgical procedure included specimens from the new-born and still-born, from infants, children, adults and old people.³ The postmortem material came from the midline above or below the umbilicus, that of the surgical clinic from amputations of the breast. Normal and pathological instances were available, including the skin of senile hyperkeratosis, and the atrophy of inanition. All specimens were warmed to 44°C., and were then injected intradermally with a mixture of dialyzed India ink and 5 per cent gelatin at 44°C. When concentrations of gelatin higher than 5 per cent were used, the finest superficial lymphatic

³ For the supply of material, we are indebted to the Departments of Pathology and of Surgical Pathology of the Cornell University Medical School and New York Hospital.

spots were completely cleared of blue color. When standard typhoid vaccine or Dick test toxin was used to excite an inflammation, similar results obtained.

In two other experiments, like that just described, four areas of inflammation were caused by intradermal injection of standard typhoid vaccine. The day after it had been introduced the inflamed regions as also two control areas of normal skin were injected with 0.01 cc. of the highly indiffusible 1 per cent pontamine sky blue in Tyrode's solution. The same immediate findings were obtained, and in one of the experiments all of the inflamed areas decolorized before the control areas did, while in the second experiment this was the case with three out of four of the inflamed areas.

In no instance did the inflammation progress; and except for a residuum of brownish pigmentation all the lesions resolved themselves within a week. In one injection following the use of typhoid vaccine and a poorly diffusible dye in which some of this latter passed subcutaneously the color persisted locally for several weeks.

It is plain from these various findings that in skin recently inflamed by heat, ultraviolet light or by bacterial vaccine or toxin, the walls of the lymphatic capillaries become far more permeable to vital dyes, irrespective of the grade of diffusibility of the latter. After intradermal injection into areas inflamed with toxins and bacterins secondary escape from the lymphatics occurs more rapidly, and the dyes are removed in much less than the time required to clear a normal area of skin of a similar injection. The alternative explanation that the dyes are changed to colorless derivatives in the inflamed region, need not be entertained since they are stable compounds. Similar though less sharp findings as concerns the rate of decolorization were obtained in the experiments upon heated skin. In our experience the inflamed skin decolorized in about one-fourth the time necessary in the case of the controls.

The Skin Lymphatics during Wheal Formation

What are the changes which take place in the lymphatic capillaries of skin stroked or otherwise stimulated to wheal formation? To test this point we have employed a subject H who responded to the usual clinical test,—a firm stroke with a blunt instrument,—by the formation of wheals which were perceptible within 2 minutes and well defined in 3. When the normal skin of this subject was stroked, the walls of the lymphatic capillaries in the affected region were found by the dye injection method to be more permeable than normal during the 2 minute latent period before appearance of the wheal.

the past von Recklinghausen (13), Gerota (14), MacCallum (15) and others have noted the extreme superficiality of the lymphatic vessels of human skin; but it seems to us that the richness of this plexus of superficial lymphatics has not been sufficiently emphasized. This has resulted from the failure of many workers to inject the channels completely. When thick dye solutions, heavy India ink suspensions and injecting masses were utilized by us to obtain permanent preparations only incomplete injections of the lymphatic capillaries resulted.

That we have been dealing with actual vessels and not artifacts was proven by the characteristic appearance of the vessels when filled with colored fluid, by the speedy drainage into lymphatic vessels high up in the arm when injections were made into living skin and by the finding of endothelial lining cells about the ink-filled channels in cut and stained sections from amputated skin. Frequently, to be sure, in making permanent preparations, the injected fluid broke out into the interstitial spaces, an occurrence readily recognizable in the gross and in cut sections, as MacCallum has already shown (15). Occasionally, too, in making an injection in skin removed from the body the needle point became lodged too superficially, among the horny cells of the corium. In these instances the injecting fluid entered the epidermis itself and spread out yielding an intercellular injection which in the gross has a rough likeness to a lymphatic injection in that the dye solution was distributed like the strands of a net. When such injections were examined under the binocular microscope, and later, when sections were examined, they could readily be recognized as spurious.

The fact that a single injection of isotonic dye solution into living skin fills the superficial lymphatic capillaries over an area 3 to 5 cm. in diameter, or more, deserves special remark for it shows as have the fixed preparations, the existence of innumerable anastomoses among those lymphatic vessels lying in the papillary stratum of the corium, vessels which escape recognition in ordinarily stained sections.

Recently Handley (16) discussing the rôle of skin lymphatics in the genesis of cancer, has stressed the anatomical relationships of these vessels as shown after injection. This author pictures small groups of six or eight blind papillary lymphatic capillaries, each group a unit as it were, meeting to form a common vessel which passes through the tela subcutanea to form with other similar vessels the subcutaneous lymphatic plexus. He holds that in the subpapillary layer of the corium only a few of these vessels anastomose and as evidence for this view (16) cites a specimen injected by Hyrtl but never

rowing or blocking their lumina combined with such an increase in the permeability of their walls that they no longer retain and pass on their contents.

The Effects of Histamine

A study was now made of the lymphatics in the wheals produced by histamine.

In some initial experiments histamine solutions containing dye were injected into normal skin, result being that the two substances were distributed together. In five experiments 1 part in 50,000 of histamine was present in an aqueous solution of patent blue V (11 per cent), injections were made simultaneously of the dye solution with and without histamine. The skin of the volar surface of the forearms of the two subjects was utilized. The injections were made 5 to 7 cm. apart, at the same level on the arm. Whealing occurred in 2 to 3 minutes with the histamine-containing dye solution. During the preliminary period and in the following 2 minutes, far more dye escaped from the lymphatics than in the localities injected with control solution. Thereafter the swelling caused by the histamine mixed with dye obscured the findings so that further comparisons were not possible. In four other experiments the test mixture consisted of histamine 1 to 100,00 and patent blue V in 5.5 per cent concentration with Tyrode's solution as the diluent to preserve isotonicity. The control solution, a 5.5 per cent solution of patent blue V in Tyrode's solution and containing no histamine was injected at the same time. Although in these instances the formation of wheals was slower, similar results obtained, showing that histamine in 1 to 100,000 increased the permeability of the lymphatic capillary wall.

In six further experiments, 1 to 50,000 watery histamine solution was pricked into a region of skin in which the lymphatic capillaries had been injected 2 minutes previously with 11 per cent, aqueous, isotonic patent blue V. As usual the procedure caused the development of a wheal, and as the wheal developed the pressure at its extending margin squeezed the colored solution along the vessels into the normal skin beyond, a course of events plainly visible under the microscope. Dye which had already escaped into the interstitial fluid and had colored the latter diffusely was also pressed out of the whealing area. The result was that the wheal became surrounded by a darkly colored ring, itself appearing pale against a dye-stained patch.

In seven experiments 0.005 to 0.01 cc. of a watery 11 per cent isotonic solution of patent blue V was injected into the skin over a freshly and fully formed histamine wheal. The dye solution passed at once into the lymphatics, was retained by them and extended along them just as in ordinary skin. The lymphatics appeared to be less compromised by pressure than in the wheals produced by stroking, and dye-stained fluid passed into and along them. During a period of 6 to 10 minutes dye solution escaped hardly at all into the interstitial fluid, but instead

scratches became visible in the local lymphatics in 1 to 3 minutes, Chicago blue appeared in them more slowly—3 to 5 minutes,—and pontamine sky blue more slowly still, in 4 to 6 minutes.

It was plain from the differences in the time required for the dye to appear in the lymphatics, this varying inversely with the diffusibility of the pigment, that the lymph channels had not been torn open by the superficial scratches. That patent blue V passed most quickly into the lymphatic stream was of course to be expected, as it was the most diffusible of the dyes used; but the fact deserves stress in the present relation since the permeability of the lymphatic capillary wall was here being tested in the direction which interstitial fluid naturally takes to enter the lymph stream, not in the reverse direction as in the previous experiments. The appearance time of Chicago blue was intermediate between that of patent blue V and pontamine sky blue, and with the aid of color filters the fact could be made out that it entered the lymph channels somewhat less slowly than the latter.

In other experiments, crystals of dye were allowed to dissolve in the fluid which exuded from the scratches. The coloring matter was soon seen in the lymph capillaries draining the lesion. Similar experiments in which crystals of dye were placed in minute punctures of the skin of the mouse ear have yielded similar results. In both the mouse and man, patent blue V invariably appeared in the venules draining the region where the crystal was dissolving. Chicago blue on the other hand appeared in the blood stream but seldom, and pontamine sky blue only when venules or capillaries had been obviously ruptured. The use of crystalline dye was not free from objection because of the marked osmotic influence of the dissolving dye. Nevertheless, it was significant that only the highly diffusible patent blue V passed into the blood stream in visible quantity. Pontamine sky blue, the most indiffusible dye that yielded positive results in the tests, the one nearest to the albumins in molecular magnitude, was drained away chiefly by the lymphatics. In human skin because of poorer visibility, the phenomenon of its entrance into the lymph stream, is not so distinctly seen as in the mouse. When a sterile, sharp needle is dipped into an isotonic solution of pontamine sky blue and the skin punctured with it, the dye almost invariably appears at once in the lymphatics close to the puncture, obviously as a result of tears in their walls.

Many such experiments, as well as those involving scarification or intradermal injection, have led us to conclude that all slight breaks in the physical barrier of the skin yield opportunity for the passage of dissolved foreign matter into the skin lymphatics, and that the slight-

the skin of the arm and thigh, the regions subjected to investigation, the superficial lymphatic network is continuous, material injected at one spot often spreading directly through the channels of a large area. The abundant anastomoses deserve emphasis because of a further finding that every least wound of the corium tears lymphatics open with result that material enters them directly. Scratches which merely abrade the epidermis without entering the corium facilitate absorption through the walls of the intact, underlying lymph channels.

To judge from our experiments the permeability of the lymphatic capillaries in the skin of man and in the ear of the mouse appears to be similar in nature and degree (2), the lymphatic wall behaving like a semipermeable membrane, irrespective of whether dye is passing in or out of the vessel. Before considering the latter conclusion as definitely established it would be desirable to carry out more work upon human skin with highly indiffusible dyes; but unfortunately the use of these leads to prolonged discoloration and hence they cannot be used freely. Since little is known about the physiological effects of the dyes actually used it has seemed best to exercise caution in their employment upon human beings, limiting the experiments so far as possible.

Slight causes suffice to increase greatly the permeability of the minute lymphatics. A stroke on the skin of an individual susceptible to whealing causes practically at once such a change in the lymphatic walls that dyes ordinarily held back by them for some time pass through at once. This is the case also when the skin has been slightly inflamed by the application of heat or ultraviolet light, and during the more considerable inflammation that results from the intradermal injection of a bacterial toxin or of killed pathogenic bacteria. How great may be the effect of very mild heat, or of moderate exposure to sunlight we are unable to say; but judging from the general similarity of the results of our experiments on man to those with the mouse already reported by us, it seems probable that such stimuli, well within the realm of the normal, may much increase lymphatic permeability.

The clinical significance of these findings is not inconsiderable. So much material enters the lymphatics when an intradermal injection is made that every injection of the sort is to a greater or less extent intralymphatic injection. In some individuals material injected in-

The rapid passage of dye along the draining lymphatics cannot have been due, under the circumstances described, to the amount of fluid injected, 1/50 cc. at each site. Nor can it be accounted for by local hyperemia with transudation from the blood vessels, for neither hyperemia nor edema had developed. One is forced to the conclusion that the lymph flow from the skin of the resting human arm is abundant. Experiments on the mouse ear have also led us to conclude that peripheral lymph movement is rapid in the skin of animals (1, 2).

The State of the Lymph Capillaries in Inflamed Skin

Much has been written upon changes of the permeability of blood vessels of the skin in response to various stimuli but the question of whether the lymphatic wall also undergoes changes seems not to have been raised. Dye injection has enabled us to observe the responses of the lymphatic capillaries under various normal and abnormal conditions.

The Effect of Heat.—In two subjects patches of skin on the forearm were heated for 1 minute by applications of a thin-walled glass chamber (2) through which water circulated at 56°C. 5 to 10 minutes thereafter a minute quantity, 0.01 cc. of a mixture of 5.5 per cent solution of Neptune blue and homologous serum, in equal parts, was injected at two points. The first injection was so placed that the dye solution filled lymphatic capillaries lying outside the heated region but included in the hyperemic flare. The second injection was made directly into the heated skin near its edge with result that dye passed into the lymphatics of the injured area and some extended into the channels outside of it.

Five experiments of the sort described were carried out on each subject. The results were the same in all. The lymphatic capillaries lying in the heated area let dye pass into the interstitial spaces far more rapidly and abundantly than did the vessels in the contiguous unheated region. In four other experiments on each subject, patent blue V, diluted to 5.5 per cent with Tyrode's solution, was used, and the same results were obtained. So, too, in tests with the less diffusible dyes, Chicago blue and pontamine sky blue. These observations, confirming as they do those in experiments on the mouse ear (2), extend the findings to human physiology.

Effect of Ultraviolet Light.—In six experiments, pieces of smooth flexible cardboard having rectangular openings measuring $1\frac{1}{2} \times 3$ cm. were strapped with adhe-

ence between the lymphatic involvement resulting from subcutaneous as contrasted with intradermal injections. Certainly in man the skin cannot be injected through ordinary hypodermic needles without entering the lymphatics directly.

The participation of the lymphatics in skin lesions must obviously be great. But it will not be susceptible of accurate analysis until more is known of the change taking place in such vessels under normal and pathological conditions. Some inferences appear justified, however. As is well known the blood capillaries in inflammatory foci become so permeable as to let out into the skin much more material than ordinarily; but in this skin the lymphatics have been so changed that they can be entered from the interstitial tissue much more readily than usual. It follows that there should occur in the inflamed area a much greater fluid turnover than usual. Our experiments show this to be the case. Dyes injected into inflamed and normal control areas of skin spread much further to begin with in the lymphatic plexus of the normal area because the walls of the channels into which the dye is introduced through tears made by the needle do not so readily let it escape into the interstitial tissue. The resulting stained patch in the normal area is broader and lighter than in the inflamed one. Nevertheless decolorization is accomplished much sooner in the inflamed area. The relative shares of the blood and lymph vessels in the process have yet to be appraised. The findings as described seem in direct contradiction to those of Menkin (20, 21) who observed that foreign materials circulating in the blood tended to localize and be held within inflamed regions, largely as the result of lymphatic thrombosis, or so he supposed. But Menkin dealt with purulent or necrotic types of inflammation and ourselves with that giving rise to no more than rubor, calor and turgor.

Under certain circumstances of turgor the lymphatics may undergo mechanical compression as we have found. This was the case in fully developed wheals due to histamine or to stroking. Quite possibly the same thing happens in sudden acute swelling due to inflammation the result being that the local accumulation of fluid from the blood persists longer than it might were the channels not pressed upon. During the development of wheals fluid escaping from the blood vessels forces from the interstitial tissue the fluid previously present therein—a fact readily demonstrated by the dye method.

vaccine 2 or 3 years previously. In both a severe skin response now followed intradermal injection of 0.05 cc. of this vaccine. Following three intradermal injections of 0.02 to 0.03 cc., given at one time on the volar surface of the forearm, axillary tenderness developed after 2 hours and 1 or 2 hours later the axillary nodes became palpable. In 12 hours inflamed lymphatic trunks could be perceived coursing from the region of injection, with hyperemic zones along them. After 22 hours headache and malaise developed.

To determine the lymph movement after intradermal injection of the several toxins used, some preliminary tests were made with pontamine sky blue or patent blue V, which were added to the injection material, using 1 volume of isotonic watery dye solution to 20 or to 10 parts of toxin respectively. In every instance, the fact could be made out that the material injected entered and coursed along the lymph capillaries like an ordinary dye solution. Pontamine sky blue was mixed with the typhoid vaccine in an attempt to determine whether the bacteria, entering a puncture, would pass into the lymphatics as well. The dye of the mixture passed directly into the lymphatic capillaries. It was impossible to recognize the bacterial bodies themselves with the available apparatus.

In the experiments proper no dye was added to the toxins or vaccines. Within 24 hours after the injection, each of the test materials had produced areas of inflammation and these were used for the experiments. Fig. 9*a*, taken from one of these, shows the erythematous macules arising from four injections of 0.01 cc. of the streptococcus toxin, photographed 18 hours and 45 minutes after inoculation. To show the erythema the photograph was taken with a Cooper-Hewitt light. The arm had been used as ordinarily since the injection. In Fig. 9*b* the same arm appears 40 minutes after an injection of 0.01 cc. of 11 per cent patent blue V into three of the four macules, M_1 , M_2 , and M_3 . Macule M_4 was left untouched as a control. Three areas of normal skin, C_1 , C_2 , C_3 , were given similar injections of dye solution at approximately the same time. Thereafter, in the time interval between the injection of dye and the taking of the next photograph for Fig. 9*c*, the arm was not rested or protected in any way but was used as ordinarily in the course of laboratory work. In the instances, Figs. 9*b* and 9*c*, the Cooper-Hewitt light was not used but the photograph was taken through an orange G Wratten filter to accentuate the blue color. As result the deep red uninjected macule (M_4) is difficult to see. Fig. 9*b* shows the injected hyperemic areas deep in color, and the injected control areas more irregular and much paler. The lymphatics in the region of inflammation proved so permeable that the injected dye passed at once from the lymph channels into the interstitial tissue to yield an even, deep coloration. The same amount of dye injected into the control areas C_1 , C_2 , C_3 , was more widely distributed through the lymph channels, escaped much more slowly into the interstitial spaces and colored the tissue irregularly.

Fig. 9*c* is a photograph of the same arm 3 hours and 10 minutes later. Already the major portion of the coloring matter has disappeared from the inflamed regions into which it was injected whereas the control areas remain heavily colored. These were not entirely free from dye 18 hours after injection by which time the inflamed

instances the lymphatic wall behaves like a semipermeable membrane. The permeability of the human lymphatic wall like that of the mouse is subject to rapid and great changes. A stroke on the skin with a blunt instrument to produce a wheal, causes the lymphatic capillaries to become so permeable temporarily that dyes pass through their walls as if practically no barrier existed, instead of being held back for a greater or less period. Slight inflammation due to heat, ultraviolet light or bacterial products has a similar effect. So, too, has histamine. When fluid pours rapidly into the tissue from the blood, as when a wheal is formed, the lymphatics are compressed and their efficiency as drainage channels is interfered with.

These facts are briefly discussed in their bearing upon skin phenomena in general. The lymphatics cannot be disregarded in considering such phenomena, in which it is plain that they have a large share.

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In six experiments, a solution of equal parts of aqueous isotonic Neptune blue and homologous serum was injected intradermally during the latent period. Wherever the dye-containing lymph channels crossed the area eventually involved in the wheal, there was dye escape from them, whereas from those outside the region of stroke no such escape occurred. These findings with human skin corroborate the results in mice (2) (see Fig. 1 of paper referred to).

Pontamine sky blue in isotonic approximately 1 per cent solution, that is to say, 0.1 cc. of isotonic 21.6 per cent aqueous dye solution mixed in 2.16 cc. of homologous serum, passes out of normal human skin lymphatics very slowly. Only after 15 or 20 minutes is extravascular color seen. This poorly diffusible mixture, injected during the latent interval after stroking, and extending along the lymphatic capillaries within the area which had been stroked, escaped from the channels in the line of stroke.

In the experiments with pontamine blue the fact was noted with the microscope that as the wheal developed it pressed the colored fluid along the channels and out of the region involved. Two intradermal injections of 0.1 cc. of isotonic 11 per cent patent blue V in Tyrode's were made into a normal area of skin on the forearm. When 4 hours had elapsed, and the dye had spread evenly through the interstitial tissue forming two colored patches across which the skin was firmly stroked to evoke a wheal. On both sides of the line of stroke wheal formation became visible 2 minutes later and in 3 minutes was well defined, with sharp margins. At this time the photograph reproduced in Fig. 8 was taken under a pool of oil, held from flowing away by plasticine placed on the skin round about. Although the definition thus obtained was excellent the phenomena were more marked to the naked eye than in the figure which does not render the color contrast in its entirety. Along each side of the relatively pallid wheal was a dark line of dye. The dye in this line lay in the interstitial tissue, not in the lymphatics and must have been pressed out of the wheal region by the edema fluid. Tests of the sort described were repeatedly made on the same subject and the results were always the same. As the wheal edema developed it compressed the lymphatics contiguous to the injury, as could be directly seen, thus narrowing or blocking the channels. This point has been demonstrated by applying patent blue V to a scratch made in the region where whealing has just started, according to the technic already described, or introducing it by means of a needle puncture. The appearance of the dye in the lymphatics in which the test was made immediately after the whealing stroke or after the wheal had begun to regress was not delayed as compared with the event in instances above.

The experiments show that the lymphatics of the skin submitted to a whealing stroke become at once abnormally permeable; that as the wheal develops fluid previously present in the region involved is forced into the surrounding skin, and that the lymphatics in the wheal region lose their effectiveness as draining channels. This last change would seem to be the result of direct pressure on the lymphatics nar-

PLATE 49

FIG. 9a. Four erythematous macules 18 hours and 45 minutes after intradermal injections of 0.01 cc. of a streptococcus toxin described in the text. To bring out the differences from the normal skin the photograph was taken with a Cooper-Hewitt mercury arc light. An old scar, directly above the antecubital fossa is to be ignored.

FIG. 9b. The same arm shown in Fig. 9a, 1 hour and 10 minutes later, and 40 minutes after an injection, of 0.01 cc. of an 11 per cent patent blue V in Tyrode's solution, into the macular areas M_1 , M_2 and M_3 . Three similar control injections were made intradermally into the normal skin, at C_1 , C_2 , C_3 . The arm has for the moment been supinated slightly for photographic purposes so that areas M_1 , M_2 and M_3 appear higher than the others. To bring out the blue color of the dye, the photograph was taken through an orange Wratten G filter with result that the uninjected macule M_4 which was actually deep red appears almost invisible.

The hyperemic areas receiving the dye are now of a deep blue color owing to its rapid secondary escape into the tissue from the lymphatic capillaries into which it had at first passed. The injected control areas are much lighter in color, for in these the dye was more widely distributed through the lymphatics before its escape.

FIG. 9c. The same arm 3 hours and 10 minutes later. The findings are now wholly different, the control areas being much the darker, owing to an almost complete decolorization of the inflamed ones.

the injected lymphatic channels became paler and were apparently cleared of dye in 12 to 18 minutes. During the regression of the wheal the dye in the lymphatics appeared to be diluted rapidly.

It would seem from these findings that the abnormal permeability of the lymphatics noted immediately after histamine injection no longer existed in the fully formed wheal. Lewis (18) in his studies of the responses of blood vessels of the skin called attention to the finger-like extensions of histamine wheals as indicative of a spread of the substance through the lymphatics, with its subsequent escape and secondary wheal formation along the course of the vessels. In some of our experiments evidence was obtained in support of this supposition.

In five instances fully formed histamine wheals with finger like projections were elicited by puncturing the skin through a drop of aqueous, histamine solution 1 to 1000 dilution. Subsequent intradermal injections of the dye were made directly into the skin overlying the wheal. In all five instances as the dye solution was carried away in the lymphatic channels vessels of this sort were disclosed passing directly along the axis of the whealed finger-like projections. It seemed clear that the histamine must have escaped from them and given rise to the secondary whealing about them.

The experiments show that lymphatic capillaries affected by histamine allow dye to pass into the interstitial spaces more readily than do those of the normal skin, a state of affairs also found in skin submitted to stroke for whealing purposes. Although histamine clearly increased the permeability of the lymphatic wall in the experiments in which the substance was injected together with dye solution, this did not seem to be the case when dye was injected into fully formed wheals. As whealing developed the fluid previously present in the skin was forced into the adjacent tissue and the lymphatics within the whealed region were compressed.

DISCUSSION

The experiments here presented show that it is possible to study directly the lymphatics of living human skin. They have demonstrated the existence of a rich plexus of minute lymph vessels in the superficial layer of the corium, a fact many times proved by others but not ordinarily taken into account in appraising the factors involved in skin reactions. Some have supposed the capillary lymphatics to be divided into small groups, each group draining into the tela subcutanea and essentially separate from the others because of the absence of superficial anastomoses. We find on the contrary that in

tradermally spreads through the superficial lymphatics of a considerable area whereas in others it is more localized, a difference due, as we have found, to differences in skin texture such that in some persons the injecting needle tends to penetrate deeper than in others, with result that material is deposited mainly beneath the lymphatic plexus. To this fact, certain of the differing individual reactions to intradermal test injections that are observed clinically, can be laid. In both types of individuals, however, injected material finds its way promptly into the large draining lymphatics. The rapidity of this occurrence when minute quantities of dye-containing solution are introduced into the skin proves that the flow along the lymphatics draining the skin is rapid even when the limb is at rest, and by corollary that fluid turnover within the skin must be abundant. Dye can often be perceived in the lymphatics at the axilla within 10 minutes after the injection of 1/50 cc. of dye-containing solution into the skin of the forearm. It follows that not only is an intradermal injection an intralymphatic one, often almost entirely so, but it is also to some extent a general injection, not merely a local one—more or less of the material introduced passing quickly along the lymphatics to the blood stream.

The work of previous authors has led them to conclude that there is almost no lymph flow from the resting limb. But they employed anesthetized creatures, animals which had not the greatly specialized skin of the human being with its abundance of blood vessels provided for highly developed functions. The fact should, however, be mentioned as bearing on our findings that a reason for the rapid passage of dye-bearing lymph to the axilla may be found in the circumstance that only a few of the lymphatics draining the forearm enter the supra-trochlear or epitrochlear glands, most of the injected dye being carried directly into the lymph channels of the upper arm. This does not alter the fact that lymph flow from the human cutis is remarkably rapid. Earlier work on the lymphatics in the ear of the mouse has led us to believe that peripheral lymph turnover in this organ as well is great (2). Working with dogs, Field and Drinker (19) reported the fact that subcutaneous injections of horse serum appeared in the lymph draining from an injected limb, but did not do so more rapidly when multiple injections were made, evidence that direct injection of the lymphatics did not occur. The point illustrates well the differ-

A further word may be said upon the process of wheal formation since the interpretation of this phenomenon well illustrates the current disregard of lymphatic functioning. The fact is well known that the minute blood vessels involved in the wheal become abnormally permeable even to the point of letting out substances which would not otherwise pass into the tissue, as for example Congo red (22). But it has not been realized that concurrently the lymphatics become more permeable and that though this is the case they, like the blood vessels, become so compressed by the wheal fluid that their efficiency as drainage channels is interfered with. Herein lies a reason why wheals become sharply delineated. However, a complete understanding of wheal formation and disappearance necessarily waits upon further knowledge.

SUMMARY

A technic is described for the demonstration of lymphatic capillaries in living skin and for their study. By means of vital dyes injected intradermally these vessels can be rendered plainly visible. They form an extraordinarily abundant anastomotic web. The least scratch, one which does not penetrate through the epidermis, gives rise to such conditions that lymphatic absorption readily takes place from the abraded surface; and so close-meshed is the lymphatic web that an intradermal injection with even the finest hypodermic needle tears some of the constituent vessels open with result that they undergo direct injection. In many individuals much of the fluid introduced at an ordinary intradermal injection, like that made in the clinic, spreads through the superficial lymphatic network, whereas in others it tends to enter the deeper lymphatics at once, the difference being due to merely physical factors determined by skin texture. Normal flow along the skin lymphatics is rapid even when the body is at rest, dye introduced into the skin of the resting forearm reaching the axilla within a few minutes. The observations make plain the fact that every intradermal injection is an intralymphatic one, often preponderantly such, while furthermore every local injection into the skin becomes within a few minutes a general one, so rapidly is the introduced material transported to the blood.

The normal permeability of the skin lymphatics of man is approximately the same as that of the mouse. Tests indicate that in both

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EXPLANATION OF PLATES

PLATE 46

FIGS. 1 and 2. Successive stages in the distribution of dye during injection of the skin of two individuals, M and H. Patent blue V in 11 per cent solution was used, 0.1 cc. of it in the subject M, 0.2 cc. in H. The photographs were selected from a moving picture film to show the course of events or further some characteristic individual differences. Magnification $\times 3/2$.

PLATE 47

FIGS. 3, 4 and 5. Enlargements of *a*, *d* and *e* of Fig. 1. These figures show the spread of dye in the rich network of lymphatics lying in the subpapillary layer of the corium. In Fig. 5 the spread has continued after removal of the injecting needle; in the upper left hand corner a pointer marks a region of such spread. Magnification $\times 5$.

FIG. 6. The edge of the injected area shown in Fig. 1, 5 minutes after the last picture was taken. The rapid escape of dye from the lymphatics has obscured their outlines in a dense blue cloud. At the margin many small lymphatics can be seen draining away the dye. Midway between these and the bleb is an area in which dye is escaping from the lymphatics into the interstitial spaces.

In the lower right corner a deep draining lymphatic is visible. It is one of those channels which lie in the subcutaneous fatty layer of the skin.

PLATE 48

FIG. 7. The results of the rapid injection of minute quantities of dye solution into the skin of M. Photograph taken 6 minutes after two simultaneous injections of 0.02 cc. of patent blue V, each injection extending for a period of seconds. At this time—but too faint to show in the photograph—dye could be seen in a draining trunk close to the axilla. Some dye escaped along the needle to the skin surface forming heavy oblong marks.

FIG. 8. The displacement of dye-stained interstitial fluid by the pressure of a wheal. 0.1 cc. of 11 per cent patent blue V in Tyrode's solution was injected intradermally in two places and 4 hours later when it had spread diffusely through the tissue, wheal formation was evoked by a firm stroke across the colored regions and the skin surface was covered with paraffin oil. 3 minutes later, the photograph was taken. The wheal appears as a broad pale band along the horizontal line of stroke. On both sides of it, there can be made out a dark line of dye-stained interstitial fluid displaced by the wheal fluid.

esses involved in bringing about this and other profound physiological changes encountered. The present work was undertaken to correlate, if possible, the described alterations in the blood with the excretion of various ions, particularly sodium, occurring during the development of adrenal insufficiency. For this purpose relatively complete balance studies have been made on dogs before and after adrenalectomy.

EXPERIMENTAL

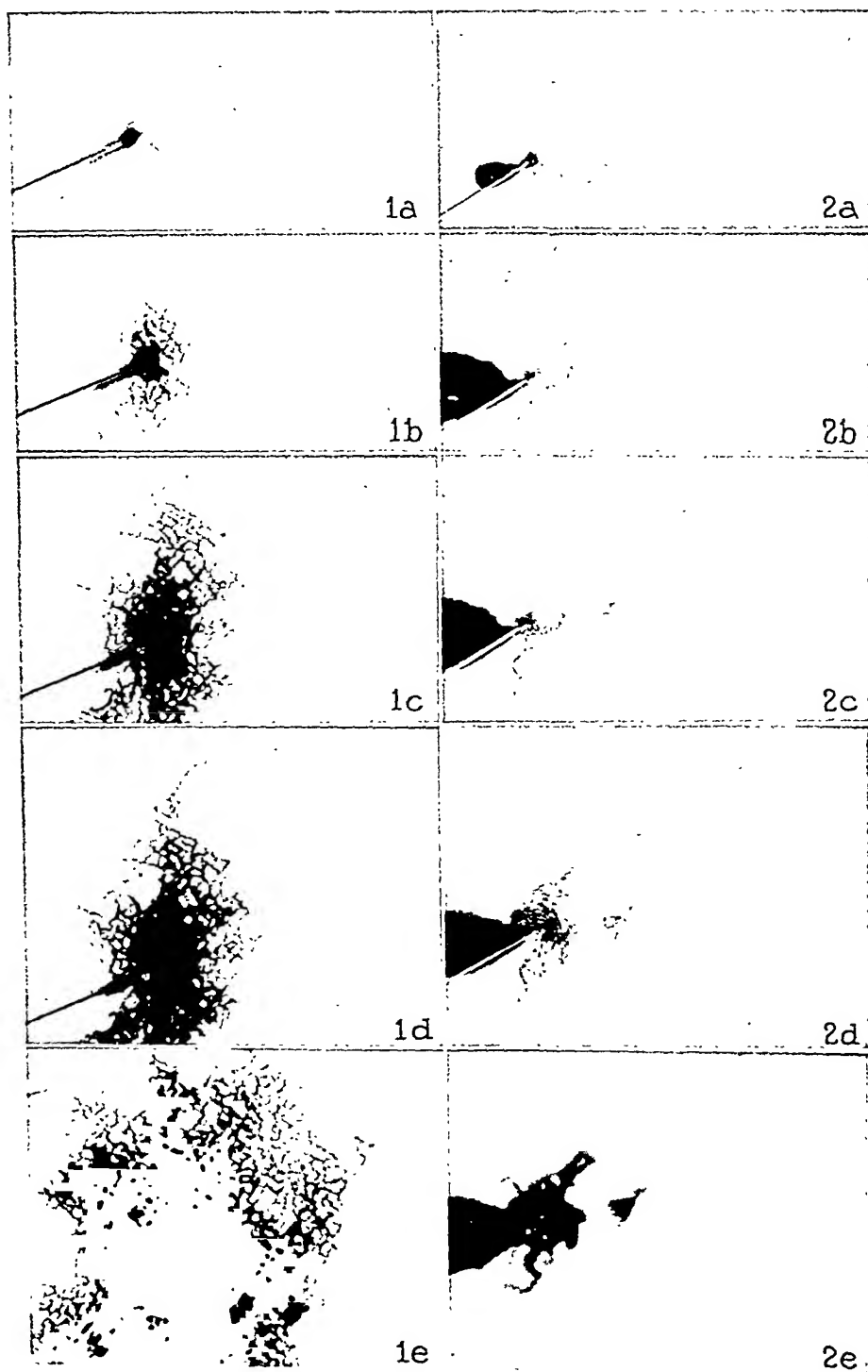
Most of the analytical methods employed have been described elsewhere (5-7). Sodium was determined according to the method of Butler and Tuthill (8). The urine, stool and food specimens used for the determination of sodium were ashed in the muffle oven at 470°C. before analysis and potassium was removed from the urine, because of its high concentration.

Adult male dogs weighing between 13 and 15 kilos served as the experimental subjects. The animals were given diets consisting solely of ground, raw, lean beef and received daily rations of 400 or 500 gm. for periods of 2 to 14 weeks before balance studies were begun. On this diet they remained in good health and their general nutrition improved. At the beginning of the observations sufficient meat to last 7 days was ground up and thoroughly mixed. The daily rations were then weighed carefully, wrapped in waxed paper and kept on ice until used. A sample of each lot of meat was dried and analyzed for total inorganic base, Na, K, Ca, Cl and total nitrogen. Water was allowed *ad lib*. The animals ate their food with relish until the 4th day after the second adrenal gland was removed, when their appetites decreased rapidly. On only one occasion was vomiting encountered in this work. Dog 2 vomited undigested meat once on the 5th day following the second adrenalectomy. The vomitus was dried and analyzed and deductions were made from the food intake.

When the experiments were started the animals were catheterized and placed in the metabolism cage, where they remained continuously. The urine was collected under toluol and at the end of each period the dogs were catheterized. The cage was scrubbed and washed with 1500 cc. of distilled water, except in the case of Dog 1, where it was merely rinsed with 500 cc. of distilled water, and these washings were added to the urine of the period. Urine specimens were analyzed for total inorganic base, Na, K, Ca, Cl and total nitrogen.

Stools were collected during each period and were usually formed. Occasionally there were soft stools and slight admixture with urine specimens resulted in these instances. This had little quantitative or qualitative effect upon the results, as the tables show that the stools contained only minute amounts of the electrolytes other than calcium. In the case of Dog 2, a little bloody mucus was passed following the second adrenalectomy. Stools were analyzed similarly to the food and urine specimens.

Blood studies were carried out on 50 to 80 cc. samples of blood removed from the



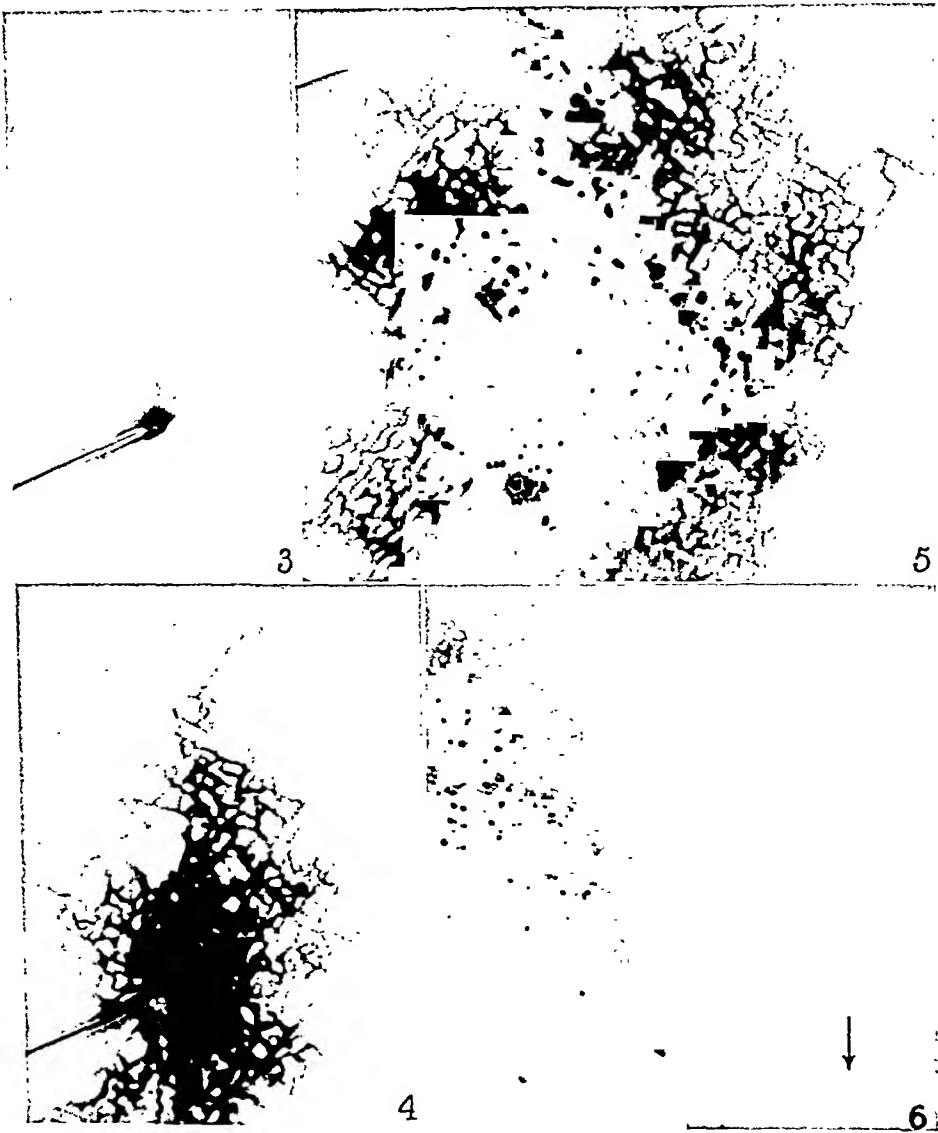
Photographed by Louis Schmidt

(Hudack and McMaster: Lymphatics of living human skin)

TABLE I
Balance Study on Dog 1

Balance Study on Dog 1

Inclusive dates	Period	Urine										Stool					Intake					Body wt.		Remarks	
		Vol.	K	Na	Ca	T.B.*	Cl	T.N	Dry Wt.	K	Na	Ca	T.B.*	Cl	T.N	K	Na	Ca	T.B.*	Cl	T.N	gm.	kg.		
1932	I																								
	Daily	332	43.5	7.9	1.49	62.3	5.2	18.1	3.7	0.12	0.20	3.6	8.6	0.1	0.19	43.2	9.3	1.1	65.3	4.6	16.2				
	Total	1990	260.7	47.3	8.94	373.6	30.9	108.5	22.0	0.71	1.21	21.7	51.5	0.5	1.13	259.2	55.8	6.6	391.8	27.6	97.2				
	II																								
	Daily	313	47.0	7.7	1.69	60.2	4.8	17.9	2.9	0.06	0.08	3.2	7.3	0.1	0.19	43.4	10.6	1.4	65.0	5.4	16.7				
	Total	2190	329.0	54.0	11.80	421.2	33.4	125.4	20.0	0.45	0.59	22.2	51.0	0.5	1.18	303.6	74.1	9.5	455.3	37.6	117.0				
	III																								
	Daily	334	114.0	5.8	1.47	123.2	65.4	17.6	4.4	0.23	0.10	6.6	13.7	0.1	0.23	112.9	10.3	1.5	135.3	72.7	17.3				
	Total	2335	798.0	40.9	10.30	862.3	457.8	123.4	31.0	1.60	0.72	46.1	95.5	0.7	1.58	790.4	72.0	10.4	946.8	503.7	121.2				5.0 gm. of KCl ingested daily with diet
	IV																								
	Daily	307	107.5	9.7	0.88	124.2	73.4	16.3	3.1	0.04	0.05	4.3	9.2	0.1	0.18	110.6	11.0	1.6	131.8	72.2	16.3				
	Total	2150	752.3	67.9	6.13	869.1	514.2	114.1	22.0	0.31	0.37	30.1	64.4	0.3	1.25	774.4	76.8	11.1	922.4	505.3	115.8				5.0 gm. of KCl ingested daily with diet
	V																								
	Daily	346	122.7	11.9	0.44	140.6	94.7	15.3	3.2	0.18	0.10	4.7	9.8	0.1	0.20	134.7	12.6	1.5	156.6	100.3	16.9				
	Total	3110	1103.9	106.9	4.00	1265.0	852.0	137.9	29.0	1.60	0.87	42.7	87.9	0.6	1.80	1212.4	113.4	13.4	1409.5	902.9	152.3				7.0 gm. of KCl ingested daily with diet. 1st adrenalectomy on 7th day of this period



Photographed by Louis Schmidt

(Hudack and McMaster: Lymphatics of living human skin)

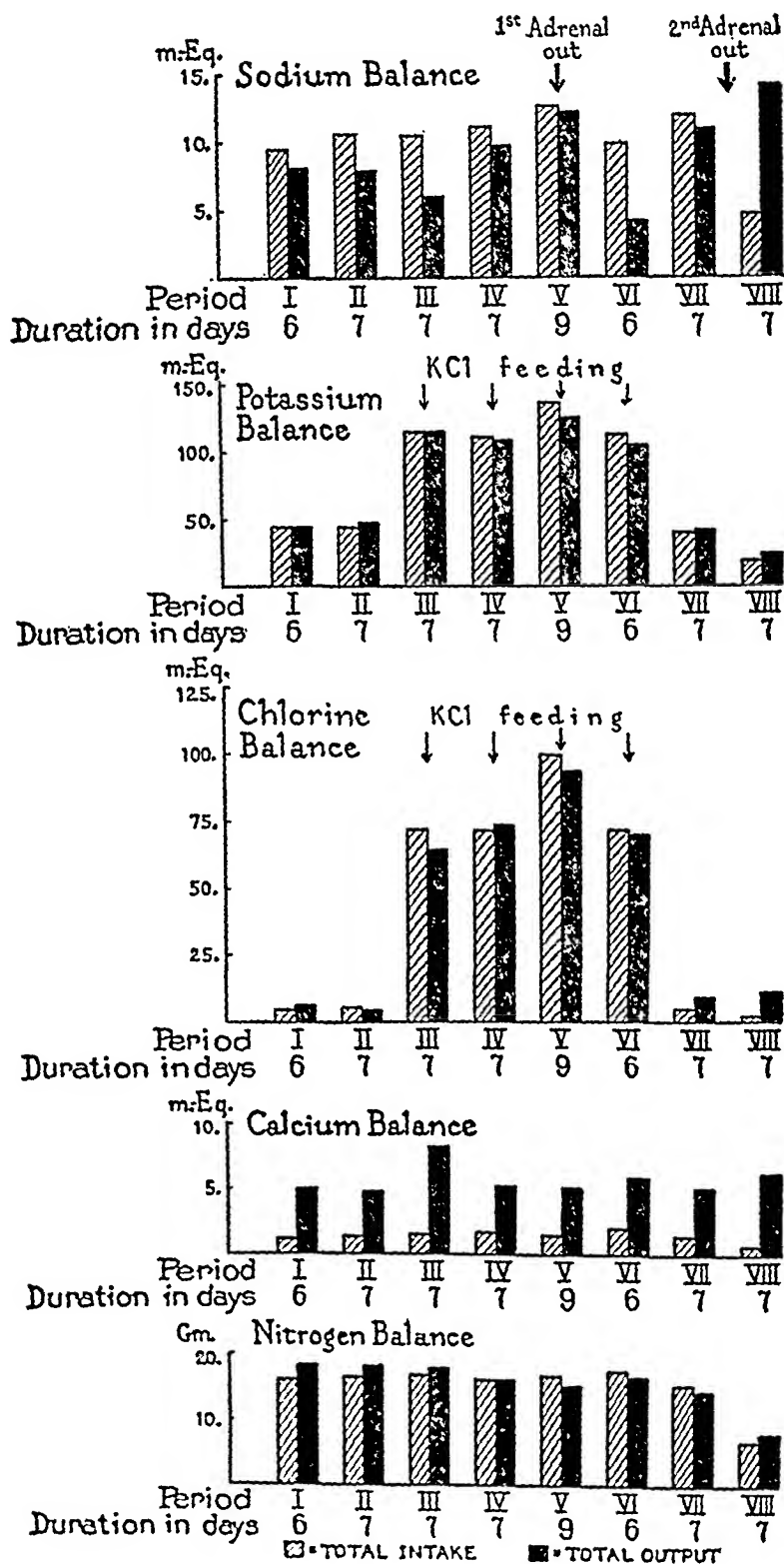
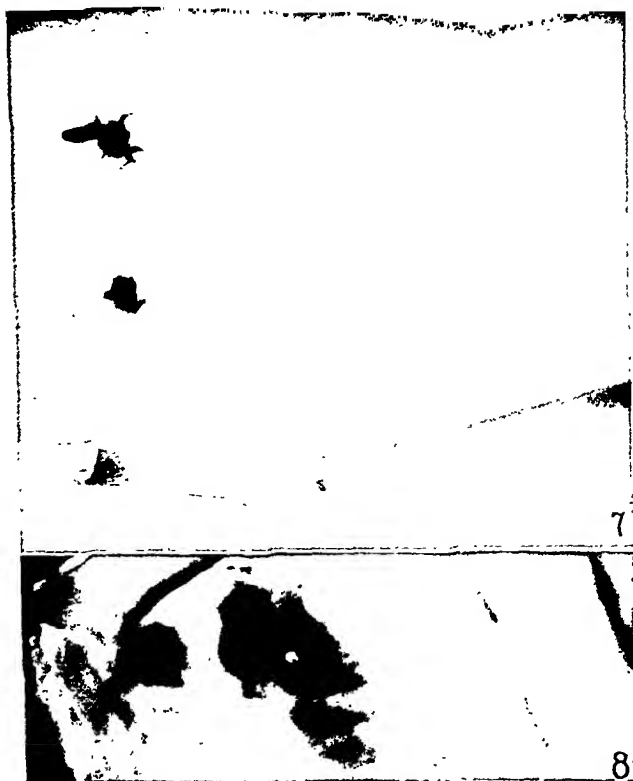


FIG. 1. D.



Photographed by Louis Schmidt

(Hedack and McMaster: Lymphatics of living human skin)

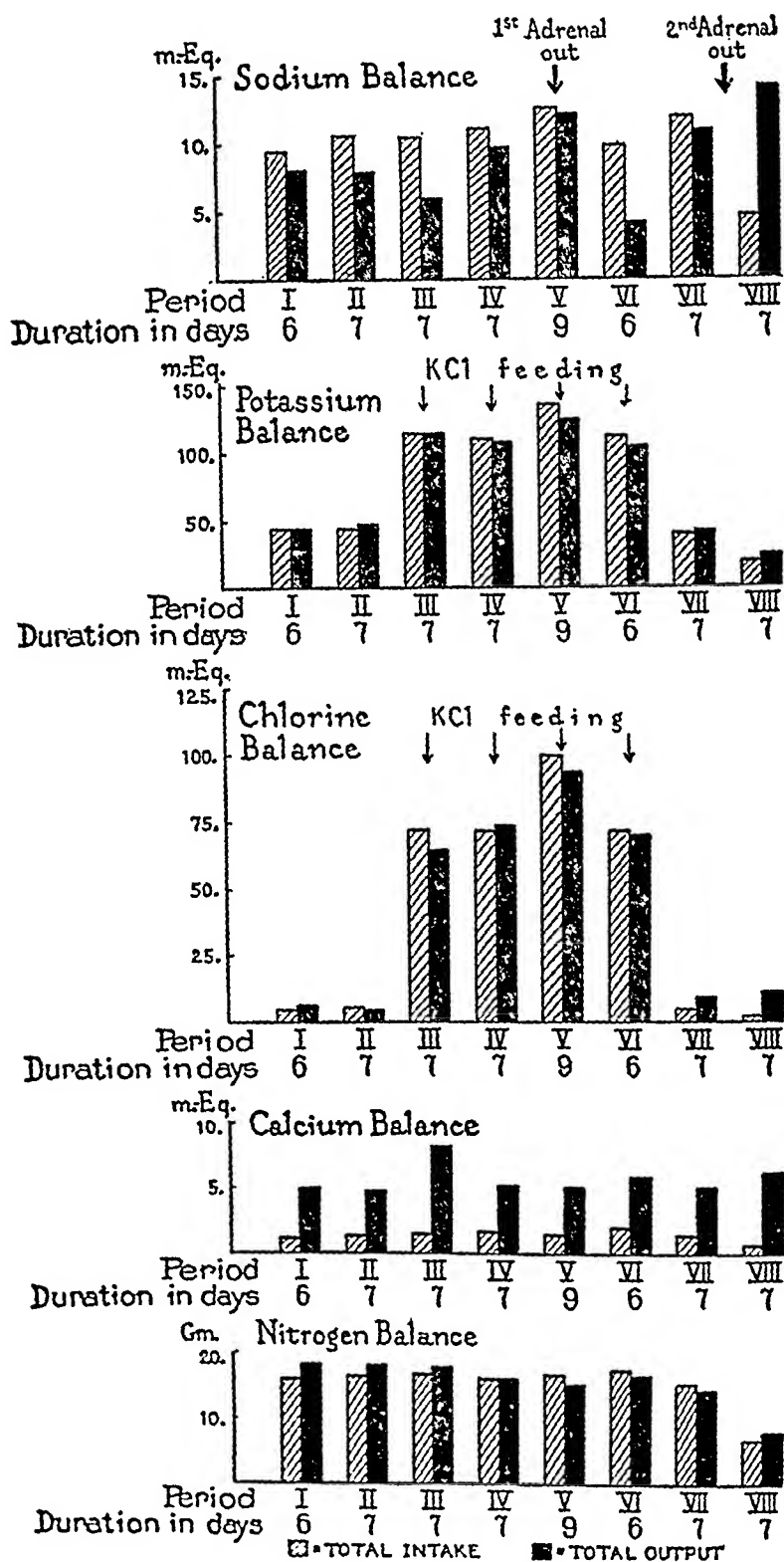
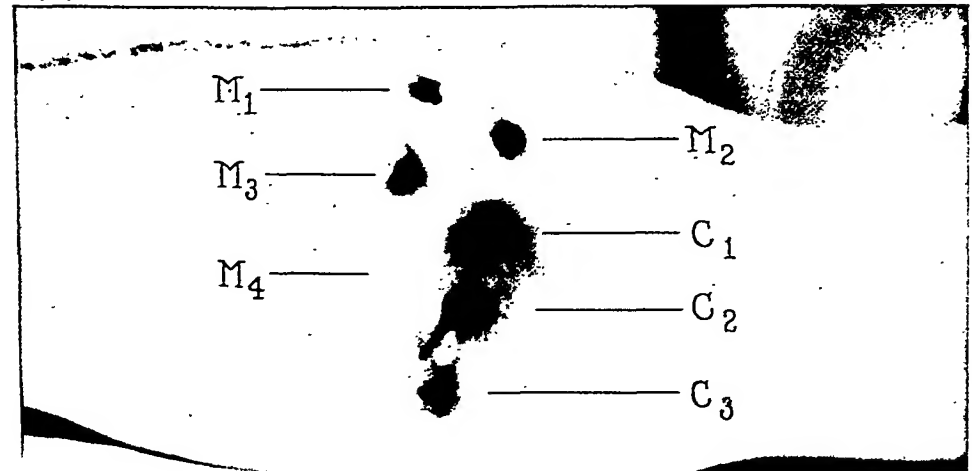


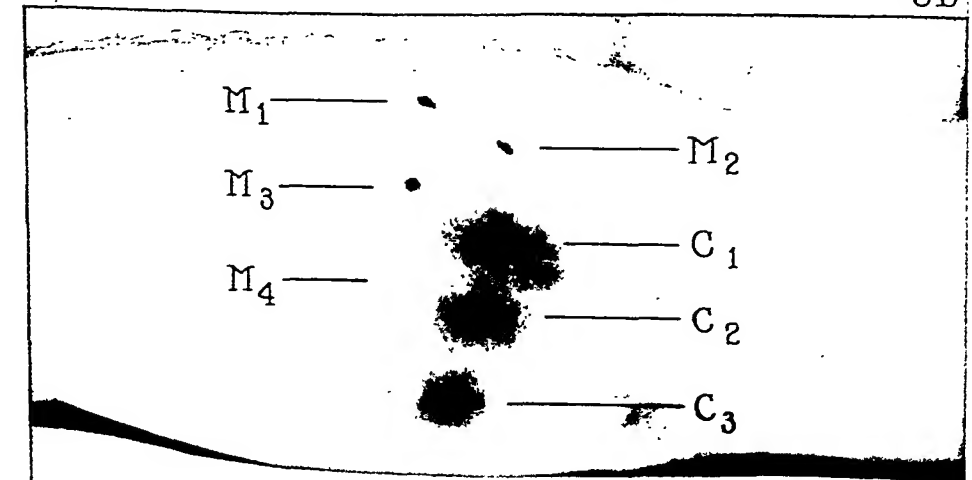
FIG. 1. B-1



9a



9b



9c

Photographed by Louis Schmidt

(Hedack and McMaster: Lymphatics of living human skin)

ELECTROLYTE BALANCE AFTER ADRENALECTOMY

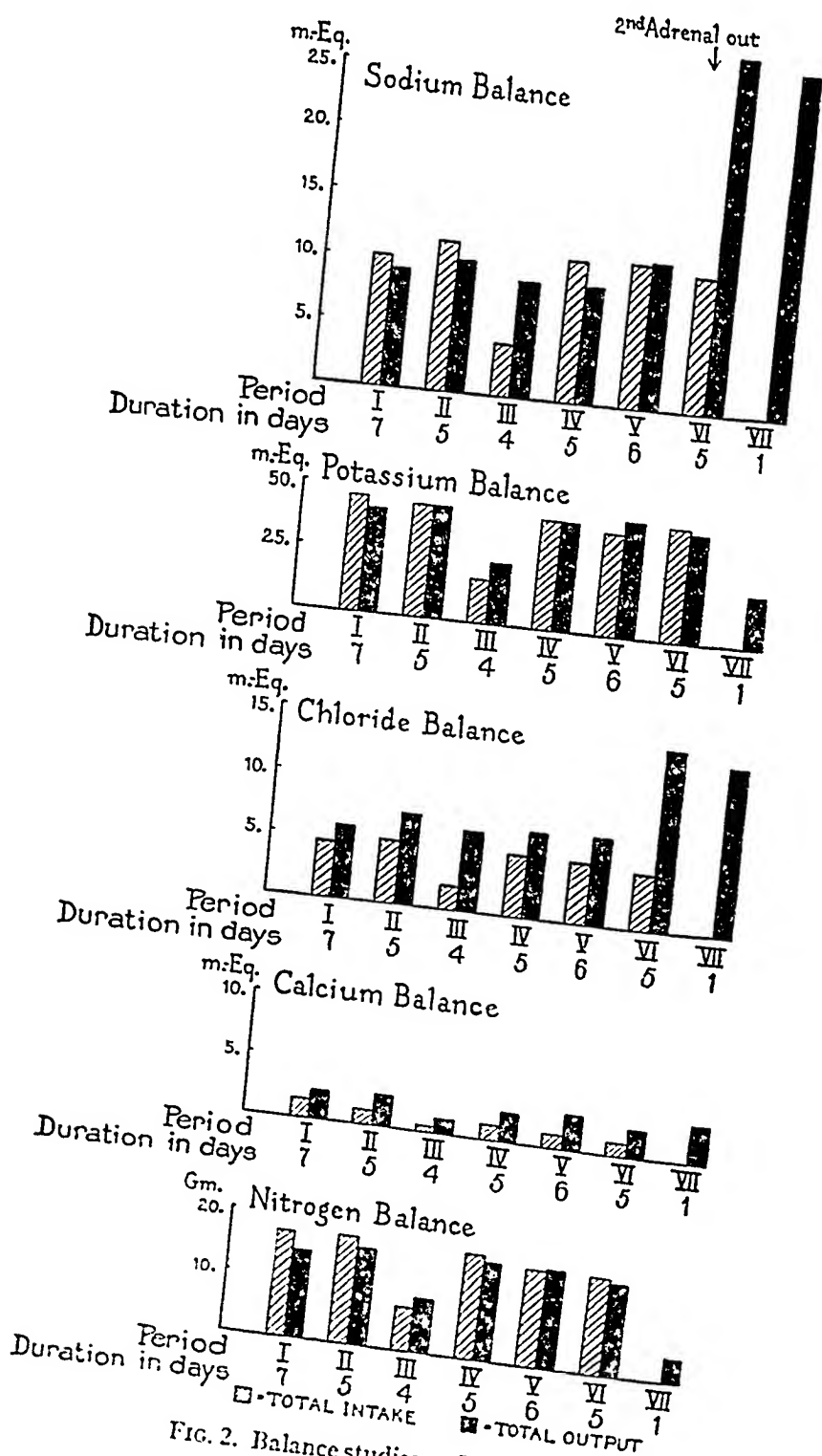


FIG. 2. Balance studies on Dog 2.

ELECTROLYTE BALANCE STUDIES IN ADRENALECTOMIZED DOGS WITH PARTICULAR REFERENCE TO THE EXCRETION OF SODIUM

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A characteristic clinical picture develops as the loss of inorganic base from the body progresses in diabetic acidosis, in severe diarrheas (11), in high intestinal obstruction and probably in certain other pathological states. The changes encountered are profound weakness, prostration, dehydration, anorexia, nausea, vomiting, fall in blood pressure, shock at times accompanied by anuria, retention of non-protein nitrogen, decrease in the concentration of the chloride ion in the blood and frequently a fall in the bicarbonate content of the serum. In view of the fact that this clinical picture is also characteristic of that present in severe adrenal insufficiency, the blood of three patients suffering from Addison's disease was studied with the result that a striking decrease in the sodium content of the blood serum was found (1). Marine and Baumann (2), in 1927, showed similarly that the sodium content of the blood of cats decreased following bilateral adrenalectomy, and suggested that sodium salts might have a "specific action" in prolonging the life of these animals. Zwemer (3) has also found, in addition to other abnormalities, a decrease in the sodium concentration of the blood of adrenalectomized cats. More recently, Harrop and Weinstein (4) have shown a slight drop in the total base of adrenalectomized dogs.

It is thus apparent that a decrease in the sodium concentration of the blood is a constant finding in both Addison's disease and in adrenalectomized animals. Since these observations have been limited entirely to the examination of the blood, nothing is known of the proc-

the food intake during Period III (4 days) was reduced from 500 gm. of meat to 250 gm. in order to note the effects of reduced food intake upon the balance. Following this, the food intake was raised to 500 gm. daily and after 11 days (Periods IV and V) the second adrenalectomy was performed. In this dog vomiting occurred once on the 5th day after operation, as was mentioned above, and the stools were small and loose. The factor of reduced diet fortunately did not occur in this dog during Period VI following the second adrenalectomy. *Sodium*: The Na balance, except in the period of reduced food intake, was constant and showed an apparent average retention of 1 m.-eq. daily. Following the removal of the second adrenal gland, the urinary Na excretion increased from an average of 10.8 m.-eq. daily to 26.9 m.-eq. and the Na lost from the body in the first 5 days after operation was 85 m.-eq. On

TABLE IV
Observations on the Blood of Dog 2

Date	Total base	Total acid	Base minus acid	Protein	Protein	Non-protein nitrogen	Cl	HCO ₃	K	Na	Remarks
	m.-eq. per liter	m.-eq. per liter	m.-eq. per liter	per cent	m.-eq. per liter	mg. per 100 cc.	m.-eq. per liter	m.-eq. per liter	m.-eq. per liter	m.-eq. per liter	
1932											
Oct. 11	157.0	142.2	14.8	5.24	12.4	16	106.4	23.4	5.1	144.6	1st adrenalectomy on Oct. 12
Oct. 25	158.0	146.0	12.0	6.17	14.6	33	107.9	23.5	5.2	146.5	2nd adrenalectomy Dec. 12
Dec. 18	144.3	128.9	15.4	6.28	14.8	185	100.0	14.1	6.3	132.8	—Died Dec. 21

the 6th day, Period VII, the Na excretion was about that of the average for the first 5 days, though the stool excretion increased and no food was taken. *Potassium*: In contrast to the increased Na excretion following adrenalectomy, there was no increase in the loss of potassium, the balance being exactly like that of the fore period. This is in agreement with the findings in Dog 1, where the negative potassium balance could be explained by reduction in food intake. *Chloride*: The behavior of the chloride excretion was, as in the case of Dog 1, qualitatively like that of the Na ion; i.e., there was an actual increase in excretion after adrenalectomy, though not equivalent to that of sodium. No explanation presents itself for the fact that this animal and Dog 3 were in slightly negative chloride balance before the second adrenalectomy. *Calcium*: The removal of the second adrenal gland did not appear to affect the negative Ca balance, present throughout the

jugular vein with oiled syringes and delivered under paraffin oil. The analyses were made in duplicate on the serum thus obtained.

Adrenalectomies were performed by Dr. Rudolph Schullinger of the Department of Surgery with ether anesthesia and with rigid surgical asepsis. In no instance was there hemorrhage or surgical shock, and all of the animals ate heartily within a few hours after the operations. Dog 3 had a superficial wound infection after the first adrenalectomy, many weeks before the period of metabolic observation began. Dogs 2 and 3 had some serosanguineous ooze from their wounds following the second operation. Salivation during anesthesia was marked and fluid thus lost was not analyzed. Autopsies were performed on the dogs and careful search revealed no evidence of accessory adrenal tissue. In Dog 2, the small intestine contained a moderate amount of "currant jelly" bloody mucus. Dog 3 showed beginning congestion of the right lower lobe.

RESULTS

Dog 1.—Balance studies were made on this animal for more than 1 month before the removal of either adrenal gland. Fig. 1 and Tables I and II show the results of this study. *Sodium:* The dog appeared to be in slightly positive Na balance (2 m.-eq. per day) during the fore period, and the irregularities observed are probably due, in part, to the fact that in this case the metabolism cage was merely rinsed and not scrubbed at the end of each period. It will be seen that the first adrenalectomy, performed on the 7th day of Period V, did not result in obvious change in Na balance when compared with the other control periods. Following the second adrenalectomy, the dog went into negative sodium balance, losing about 65 m.-eq. in the course of a week, whereas the average positive balance in the fore periods would have been about 14 m.-eq. in the same period of time. *Potassium:* The experiment on this dog was complicated by the attempt to increase the excretion of Na by the administration of large amounts of KCl. During Periods III and IV the animal was fed 5 gm. of KCl daily with its ration of 500 gm. of lean meat. During Period V this was increased to 7.0 gm. and in Period VI the dose was again reduced to 5.0 gm. That this feeding of KCl had no effect upon Na excretion is obvious from the data. Following the second adrenalectomy, the excretion of K decreased in contrast to the increasing Na excretion, but was nevertheless greater than the intake. *Chloride:* The chloride excretion in this animal was naturally complicated by the KCl administration, but it may be seen from the levels of the control periods after the second adrenalectomy. *Calcium:* This animal, as well as the other dogs, was in constant negative Ca balance, as might be expected as a result of the meat diet. The effect of adrenalectomy on the calcium balance is not apparent, and if existent is masked by the inaccuracy of stool collections. *Nitrogen:* Approximate nitrogen balance was maintained throughout the experiment, even during Period VIII when the intake was reduced following the second adrenalectomy. *Water:* During the first 4 days of Period VIII, following

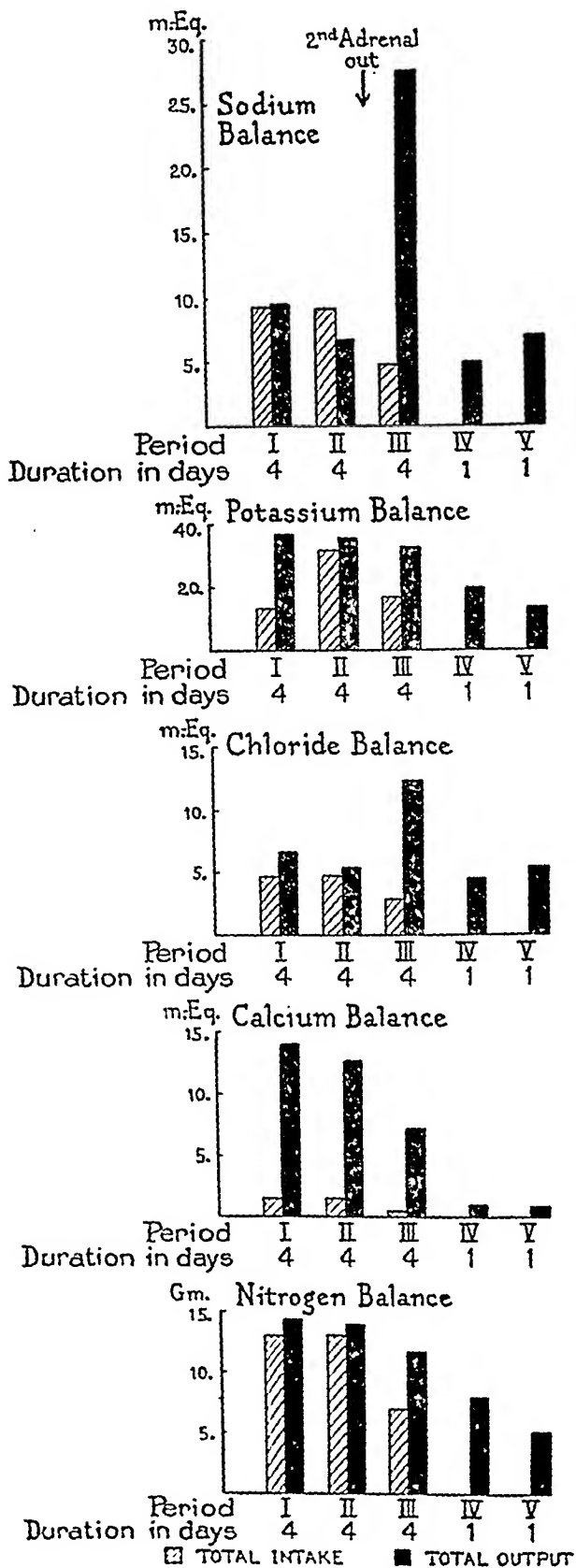


FIG. 3. Balance studies on Dog 3.

parathyroid glands upon Ca and P metabolism. In other words, one function of the adrenal glands may be to control the concentration of Na in the blood and tissue fluids. The second possibility seems untenable, because it has been shown in this work (Fig. 4) that the relative loss of Na from the body is notably greater than the loss of water after adrenalectomy. Furthermore, with primary dehydration one would expect an increase rather than the established decrease in the concentration of Na in the blood under these circumstances. The commonly known acids produced in the body in amounts sufficient to cause the extreme loss of Na described above are the ketone acids and lactic acid. The ketone acids are not known to be present in adrenal insufficiency and the lactic acid content of the blood of dogs suffering from hypoadrenalism has been shown by Harrop (4) to be unusually low. In the work here presented comparisons of total base and total acids in the blood serum gave no evidence of any significant accumulation of abnormal acid radicals. Sulfate (1) and phosphate ions increase with the retention of non-protein nitrogen, but they can hardly be held responsible for such extreme losses of Na in the urine. Thus it appears that the loss of a primary regulatory mechanism is the most satisfactory hypothesis to explain the behavior of the base sodium in adrenal insufficiency. From the evidence presented it is difficult to ignore the possibility that this assumed influence of the adrenal glands may be exerted upon renal function.

The retention of the K ion in adrenal insufficiency is of interest because this ion in normal subjects and in certain nephritic individuals has been shown (7) to pass through the kidney with greater facility than either the chloride or sodium ions.

It has been pointed out that the retention of non-protein nitrogen occurs in certain disease states in which loss of inorganic base and chloride is marked. Retention of non-protein nitrogen has also been repeatedly observed both clinically and experimentally in adrenal insufficiency, and the work here reported confirms this finding. A current explanation for this apparent kidney insufficiency has been based upon the idea that water available for urea excretion is reduced because of dehydration. Fig. 4, however, indicates clearly that during the period of nitrogen accumulation after adrenalectomy, the rate of water excretion was actually greater than that of the fore period.

the second adrenalectomy, the urine volume was augmented from a daily average of 315 cc. in the fore period to 405 cc. Thereafter, when the animal became severely ill and took neither food nor fluid, the volume fell sharply. This accounts for the fact that the daily volume as presented in Fig. 4 is less following adrenalectomy than before, in contrast with the other animals in which the periods presented in the figure were closed before the animals reached a moribund state. Fig. 4 shows that in addition to the actual increase in sodium excretion following removal of the second adrenal gland there is also an increase in relation to the water output. No such change was noted as a result of the operative procedure at which the first gland was removed. *Blood*: Numerous studies of the blood were made (Table II) and show no significant variations before the second adrenalectomy.

TABLE II
Observations on the Blood of Dog 1

Date	Total base	Total acid	Non-protein nitrogen	Protein	Protein	HCO ₃	Cl	K	Na	Remarks
	m.-eq. per liter	m.-eq. per liter	mg. per 100 cc.	per cent	m.-eq. per liter	m.-eq. per liter	m.-eq. per liter	m.-eq. per liter	m.-eq. per liter	
1932										
Sept. 20	162.3	154.1	28	7.86	18.6	26.1	109.4	5.2	148.0	
Sept. 28	158.5	152.8	30	7.34	17.3	26.5	109.0	4.5	149.0	
Oct. 6	156.3	149.0	37	6.58	15.5	24.7	108.8	4.5	146.6	
Oct. 19	158.8	150.2	31	6.74	15.9	25.0	109.3	4.8	147.9	1st adrenalectomy on Oct. 20
Nov. 2	156.3	149.7	26	6.78	16.0	25.2	108.5	4.9	147.3	
Nov. 11			214	7.31	17.3		88.8	15.4	127.5	2nd adrenalectomy on Nov. 4. Last blood taken after death

The last blood sample was unfortunately taken from the right heart shortly after death on the morning of the 8th day after operation. The changes are all similar to those seen in the other animals, except that the K content of the blood was greatly increased. As the blood was slightly hemolyzed and was taken after death, this determination is of little value. The drop in Na and Cl concentrations was 20 m.-eq. per liter, and was accompanied by an increase in non-protein nitrogen to 214 mg. per 100 cc. *Stools*: Analyses of the fecal matter after adrenalectomy failed to reveal changes from those of the fore period and the actual amounts of Na, K and Cl excreted were minimal.

Dog 2.—The first adrenal gland was removed from this animal 1 month before the metabolic observations were begun and it was kept on the standard meat diet for 3 weeks before the beginning of the balance study, the results of which are shown in Fig. 2 and Tables III and IV. During the fore period, which lasted 27 days,

parathyroid glands upon Ca and P metabolism. In other words, one function of the adrenal glands may be to control the concentration of Na in the blood and tissue fluids. The second possibility seems untenable, because it has been shown in this work (Fig. 4) that the relative loss of Na from the body is notably greater than the loss of water after adrenalectomy. Furthermore, with primary dehydration one would expect an increase rather than the established decrease in the concentration of Na in the blood under these circumstances. The commonly known acids produced in the body in amounts sufficient to cause the extreme loss of Na described above are the ketone acids and lactic acid. The ketone acids are not known to be present in adrenal insufficiency and the lactic acid content of the blood of dogs suffering from hypoadrenalism has been shown by Harrop (4) to be unusually low. In the work here presented comparisons of total base and total acids in the blood serum gave no evidence of any significant accumulation of abnormal acid radicals. Sulfate (1) and phosphate ions increase with the retention of non-protein nitrogen, but they can hardly be held responsible for such extreme losses of Na in the urine. Thus it appears that the loss of a primary regulatory mechanism is the most satisfactory hypothesis to explain the behavior of the base sodium in adrenal insufficiency. From the evidence presented it is difficult to ignore the possibility that this assumed influence of the adrenal glands may be exerted upon renal function.

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TABLE III
Balance Study on Dog 2

Balances Given on Dec 8

Inclusive dates	Period	Urine						Stool						Intake						Body wt. kg.	Remarks
		Vol.	K	Na	Ca	T.B.*	Cl	T.N	Dry wt.	K	Na	Ca	T.B.*	Cl	T.N	gm.	gm.	gm.	gm.		
1932	I	cc.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	gm.	gm.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.	m.-eq.
	Daily	295	40.4	8.3	0.66	55.7	5.84	14.3	3.4	0.20	0.73	1.70	6.24	0.14	0.35	44.0	9.96	1.47	63.8	4.52	16.9
Nov. 11-20	Total	2065	282.9	58.4	4.59	390.4	10.91	100.0	25.0	1.42	5.12	11.91	43.71	0.95	1.73	308.0	69.72	10.29	446.8	31.64	118.3
Nov. 23-27	II	336	42.9	9.2	1.28	62.1	7.05	15.9	2.6	0.41	0.90	1.23	5.09	0.36	0.24	43.7	11.36	1.36	69.4	5.00	17.6
	Total	1680	214.7	45.8	6.42	310.2	35.30	79.5	13.0	2.05	4.48	6.16	25.46	1.78	1.20	218.5	56.80	6.80	347.0	25.00	88.0
Nov. 28-Dec. 1	III	186	23.4	8.6	0.67	38.5	6.15	8.9	1.5	0.14	0.21	0.57	—	0.05	0.13	16.7	3.97	0.52	26.9	1.86	6.9
	Total	742	93.6	31.3	2.68	153.8	24.60	35.6	6.0	0.55	0.84	2.33	—	0.21	0.53	66.8	15.88	2.08	107.6	7.44	27.7
Dec. 2-6	IV	332	42.6	8.5	0.93	63.7	6.82	16.1	2.8	0.15	0.62	1.41	4.29	0.03	0.23	42.7	10.82	1.35	70.0	4.94	17.2
	Total	1660	212.8	42.7	4.61	318.6	31.10	80.8	14.0	0.73	3.09	7.03	21.43	0.16	1.15	211.7	54.11	6.75	349.9	24.69	85.8
Dec. 7-12	V	281	42.7	10.1	0.77	62.9	6.92	15.7	4.0	0.51	0.92	2.15	7.21	0.24	0.32	40.0	11.12	1.28	66.5	4.86	15.7
	Total	1690	256.0	62.4	4.62	377.6	41.50	91.2	21.0	3.07	5.51	12.88	43.28	1.42	1.92	240.0	66.73	7.70	398.8	29.14	91.1
Dec. 13-17	VI	156	40.4	26.9	0.65	77.4	13.9	14.5	4.6	0.41	0.45	1.76	6.86	0.24	0.76	42.7	10.40	1.20	62.3	4.28	15.7
	Total	2280	201.9	134.5	3.27	387.0	69.5	72.5	23.0	2.01	2.26	8.81	34.31	1.19	3.80	213.8	52.02	6.00	311.3	21.40	78.7
Dec. 18	VII	345	19.0	21.7	1.82	51.1	13.2	4.84	4.0	0.66	1.79	1.36	—	0.72	—	0.0	0.0	0.0	0.0	0.0	0.0
	Total	315	19.0	24.7	1.82	51.1	13.2	4.84	4.0	0.66	1.79	1.36	—	0.72	—	0.0	0.0	0.0	0.0	0.0	0.0

* T.B. = total inorganic base.

(10) mouse typhoid, and the susceptible lines suffered a higher mortality than the resistant lines when exposed to herd infections (10, 11). In addition, the progeny of individuals surviving experimental *B. aertrycke* mouse typhoid proved more resistant to this infection and to mercury bichloride poisoning than the progeny of unselected mice, and conversely, the progeny of individuals succumbing early to the infection proved more susceptible to mouse typhoid and to bichloride poisoning (12).

The experiments suggested, therefore, that there are inherited factors in resistance which affect the course of infection profoundly.

A study of the relative importance of inherited and acquired factors in determining the outcome of infection has now been undertaken. In preface to this, it was necessary to determine quantitatively, if possible, the relative amounts of resistance inherent in given animal lines. The present paper deals with the findings. By repeated selections from the hybrid Rockefeller Institute line of albino mice, which showed a 37.4 per cent mortality to natural *B. enteritidis* mouse typhoid infection under standardized conditions, lines with approximately 15 and 85 per cent mortalities respectively were developed, and by selections from numerous other closely inbred lines of mice, two lines, with mortalities of 89.0 and 97 per cent, were obtained. A line with 89 per cent fatality, when mated with a line with 15 per cent fatality, gave F_1 progeny with 17.7 per cent mortality, which when back-crossed with the high and low mortality lines gave evidence of segregation of susceptibility and resistance factors according to heredity. It was demonstrated, furthermore, that under these controlled conditions the response of animals to the infectious organisms was determined by the amount of resistance which they had inherited. The findings will now be described in detail.

Technique

Source of Mice.—Rockefeller Institute, white-face, black-and-tan, black, spotted, agouti, hairless, Bagg, dwarf, and pink-eye strains have been used in this study. The R. I. mice came from the main Rockefeller Institute breeding room; the others were obtained by M. R. Irwin from the Bussey Institution. The former have been inbred for 18 years; the latter brother and sister inbred for at least 12 generations. All were tested several times for the presence of mouse typhoid bacilli in their stools before being admitted to the breeding room.

Maintenance of Breeding Room.—The breeding room isolated from other animal quarters is used exclusively for the rearing of these mice. The attendants devote

experiment. *Nitrogen*: As in the case of Dog 1, nitrogen equilibrium was maintained during the first 5 days after operation, while the dog was still able to eat. *Water*: It may be seen from Fig. 4 and Table III that during the first 5 days following adrenalectomy the daily urine volume increased over the daily volume of the fore period. Nevertheless, the concentration of Na in the urine actually increased as it did in Dog 1. *Blood*: The sample of blood taken on the 6th day after the second adrenalectomy showed striking changes from the analyses made before and after the first adrenal gland had been removed (Table IV). The total inorganic base decreased 13 m.-eq. per liter, the Na concentration fell 12 m.-eq. per liter and the K increased 1 m.-eq. per liter. The fall in Na was accompanied by a loss of 7 m.-eq. per liter of chloride and about 9 m.-eq. in the concentration of bicarbonate. The non-protein nitrogen increased from a normal level to 185 mg. per 100 cc. and this occurred during a period of relative diuresis. The serum protein concentration increased but slightly. The fact that the difference between the total inorganic base of the blood serum and the summation of those acid radicals determined by analysis increased only slightly after the second gland was removed, suggests that the loss of Na was probably not the result of acidosis.

Dog 3.—The right adrenal gland was removed from this animal almost 3 months before the period of metabolic study was begun. It was given a standard diet of 500 gm. of lean meat 2 weeks before the collection of specimens was started and 6 days before the experiment began, this ration was decreased to 400 gm. daily and was maintained at this level. The dog was almost moribund on the morning of the 7th day after the second adrenalectomy and was chloroformed after the last sample of blood had been taken. This animal did not vomit and there was no diarrhea until the 5th day after the second operation. The data obtained from the study of this dog are presented in Fig. 3 and in Tables V and VI. *Sodium*: As in the other dogs, there was a striking increase in the sodium excreted in the urine during the first 4 days following the second adrenalectomy (Period III). The daily balance changed from an average of +1.1 m.-eq. in the fore period to -22.6 m.-eq. as insufficiency developed. The total loss of Na from the body in these 4 days amounted to 90.4 m.-eq. During the next 2 days when the dog had stopped taking nourishment, and after 80 cc. of blood had been removed on each of the 2 successive days, the Na excretion fell to 5.0 m.-eq. and 7.0 m.-eq. respectively. *Potassium*: As in the other dogs, there was no increase in K excretion following the second adrenalectomy, although the balance was negative, again probably resulting, at least in part, from the decrease in food intake. *Chloride*: Following the removal of the second adrenal gland, there resulted, as in Dogs 1 and 2, an increase in Cl excretion as compared with the fore period, and in this animal, too, it will be seen that this loss is not as great as that of sodium. Chloride excretion decreased during the last 2 days of life, as did the elimination of the sodium ion. *Calcium*: The negative Ca balance present in this animal is even more striking than in the other dogs and this quantitative difference might perhaps be explained by the fact that it had received a pure meat diet for a shorter time before

observed carefully, and kept under uniform conditions at ice box temperature. In so far as can be determined, the strain has not altered.

It is important to restate that the colony of about 3,500 mice is continually tested for the presence of mouse typhoid and other intercurrent infections. The progeny of susceptible, resistant, and other lines are housed together at random, six to eight per cage, after weaning, for at least 4 weeks, and no infection takes place. Many samples of normal mice are sacrificed and cultured at autopsy. Carrier tests on all breeding stock are made as routine. The death rate of mice more than 1 week old is less than 0.1 per cent per month. All mice found dead are autopsied and cultured. The stock has apparently remained free of infection.

Maintenance of Unselected Control Mice.—Housed with selected mice and kept under identical conditions are the breeding stock and litters of the unselected R. I. mice. Some are bred brother to sister; some are pen inbred.

Technique of Selecting Additional Susceptible Lines.—Mice from the Bussey Institution were bred brother and sister until sufficient accumulated for testing. Selections were made according to the results of the progeny test. The mice were tested at the same time as the selected and control R. I. mice with the same culture and under the same conditions.

Selection of Susceptible and Resistant Lines

Table I and Text-fig. 1 show the crude data of per cent mortality of twenty-six tests on a total of 771 unselected, 2,942 selected susceptible, and 2,265 selected resistant Rockefeller Institute mice. In each test the sets of mice, brought up under identical conditions and of the same age, received the stated dilution of the same culture. Prior to August, 1931, all received the same dose; later the susceptible batches received a smaller and the resistant a larger dose than the unselected batches. The only known variable which could account for the differences in mortality is heredity. The seventeen tests on the unselected mice given 5,000,000 *B. enteritidis* resulted in a mean mortality with its standard error of 37.4 ± 1.6 per cent. The value of L according to the method which Irwin has described (7), as developed from Lexis, Fisher, and Wallace and Snedecor, is 0.932, and χ^2 and P according to Fisher (13), 14.8 and 0.55, indicating that the differences in results of different tests conform to normal expectancy and that a suitable control of variables in the technique had been achieved.

In each test the mortality of the unselected mice was less than that of the selected susceptible and greater than that of the selected resistant mice. The ranges of mortality of the different lines overlap slightly but the differences as judged by the sum of χ^2 s of tests are

TABLE V
Balance Study on Dog 3

Inclusive dates	Period	Urine						Stool						Intake						Body wt. kg.	Remarks			
		Vol. cc.	K m.-eq.	Na m.-eq.	Ca m.-eq.	T.B.* m.-eq.	Cl m.-eq.	T.N. gm.	Dry wt. gm.	K m.-eq.	Na m.-eq.	Ca m.-eq.	T.B.* m.-eq.	Cl m.-eq.	T.N. gm.									
1933 Jan. 10-13	I																							
	Daily	276	36.3	9.0	1.66	54.4	6.6	13.9	7.5	0.01	0.43	12.2	22.1	0.11	0.48	33.5	9.16	1.56	50.6	4.80	13.1	15.0		
	Total	1105	145.3	36.1	6.64	217.4	26.2	55.7	30.0	0.15	1.73	48.9	88.2	0.42	1.92	131.0	36.61	6.24	202.4	19.20	52.4	14.7		
Jan. 11-17	II																							
	Daily	281	35.5	6.4	1.85	52.7	5.3	13.3	8.3	0.01	0.36	10.8	19.1	0.14	0.55	33.5	9.16	1.56	50.6	4.80	13.1			
	Total	1135	112.1	25.5	7.38	210.6	21.2	53.1	33.0	0.14	1.43	43.0	76.4	0.56	2.18	131.0	36.61	6.24	202.4	19.20	52.4			
Jan. 18-21	III																							
	Daily	353	32.4	27.1	0.95	65.3	14.4	11.5	4.3	0.02	0.27	6.1	8.9	0.08	0.32	17.2	4.76	0.57	29.8	2.88	6.9		2nd adrenal- ectomy at the begin- ning of this period	
	Total	1410	129.5	108.5	3.81	261.1	57.7	46.3	17.2	0.08	1.08	24.4	35.5	0.33	1.27	68.8	19.01	2.28	119.2	11.52	27.6			
Jan. 22	IV																							
	Daily	275	19.4	5.00	1.06	29.8	4.7	7.24																
	Total	275	19.4	5.00	1.06	29.8	4.7	7.24																
Jan. 23	V																							
	Daily	210	13.2	7.00	0.79	25.5	5.6	5.27																
	Total	210	13.2	7.00	0.79	25.5	5.6	5.27																

* T.B. = total inorganic base.

TABLE I

Comparative Mortalities of Unselected and All Selected Susceptible and Resistant Rockefeller Institute Lines Following Intrastomachal Instillation of B. enteritidis Mouse Typhoid

Date	Unselected mice				Susceptible mice				Resistant mice			
	Dose	No. injected	No. dead	Dead	Dose	No. injected	No. dead	Dead	Dose	No. injected	No. dead	Dead
1930				per cent				per cent				per cent
Aug.					6*	106	48	45.2	6	23	1	4.3
Sept.					6	267	133	49.8	6	6	0	0.0
Oct.					6	229	121	52.8	6	16	0	0.0
Nov.					6	217	100	46.0	6	29	4	13.8
Dec.	6	52	21	40.3	6	167	68	40.7	6	24	8	33.3
1931												
Jan.	6	45	17	37.7	6	27	14	51.8	6	31	3	9.6
Feb.	6	55	12	21.8	6	48	35	72.9	6	51	7	13.7
Mar.	6	53	19	35.8	6	111	97	87.3	6	67	7	10.4
May 5					6	278	256	92.0	6	122	14	11.5
8					6	139	122	87.7	6	136	18	13.2
June	6	26	10	38.5	6	143	115	80.4	6	121	11	9.1
July	6	29	9	31.0	6	6	6	100.0	6	18	3	16.7
					5	99	69	69.6	7	67	8	11.9
Aug.	6	50	18	36.0	6	44	39	88.6	6	29	2	6.9
					5	86	70	81.3				
									7	56	3	5.3
Sept.	6	21	8	38.0	4	67	37	55.2	7	100	9	9.0
Oct. 1	6	47	15	31.9	4	23	8	34.7				
									7	133	8	6.0
15					5	162	122	75.3	7	139	22	15.8
Nov.	6	48	17	35.4	5	154	105	68.1				
									7	151	37	24.5
Dec.	6	50	26	52.0	5	59	47	79.6	7	171	16	9.3
1932												
Jan.	6	50	17	34.0	5	76	53	69.7	7	52	16	30.7
Feb.	6	51	18	35.2	6	26	25	96.1	6	48	8	16.6
Mar.	6	50	24	48.0	5	93	64	68.8	7	8	1	12.5
Apr.	6	47	16	34.0	5	154	105	68.2	7	201	29	14.4
May	6	50	22	44.0	5	44	31	70.4	7	152	36	23.7
June	6	47	19	40.4	5	117	86	73.5	7	114	25	21.9
Totals...		771				2,942				2,265		

* 5×10^6 .

Swingle *et al.* (10) have recently stated that the accumulation of non-protein nitrogen is secondary to the fall in blood pressure and the consequent failure of renal filtration. The results of our experiments, as pointed out above, show that this hypothesis is untenable.

CONCLUSIONS

1. Balance studies have been made on three dogs before and after adrenalectomy, performed in two stages.

2. It has been shown that the sodium concentration of the blood decreases in adrenalectomized dogs, as is true in patients suffering from Addison's disease and in cats experimentally adrenalectomized.

3. There are also decreases in the chloride and bicarbonate concentrations which together are approximately equivalent to the decrease in sodium.

4. An increase in the potassium concentration of the blood occurs after adrenalectomy, as reported in other studies. There is no obvious correlation of this change with changes in potassium balances.

5. The balance studies show a striking loss of sodium from the body during the development of adrenal insufficiency. This loss of Na results from an increased excretion of sodium in the urine and is not complicated by loss of base as a result of vomiting or diarrhea.

6. Following adrenalectomy, both the total amount of sodium and its concentration in the urine are markedly increased. This increase in concentration of sodium occurs in spite of an augmented urine volume.

7. The behavior of the chloride ion following adrenalectomy parallels that of the sodium ion, but the loss is not equivalent.

8. During the period of accumulation of non-protein nitrogen in the blood, the rate of water excretion by the kidney is even greater than before removal of the adrenal glands.

9. The possibility of a regulatory effect of the adrenal glands upon sodium metabolism and renal function has been discussed.

The writers are indebted to Miss Blanch Baxter, Miss Natalie Bryan, Miss Marjorie Clark, Miss Evelyne Krueger and Miss Ruth Jillson for their technical assistance in this work.

the several lines ranged from 87.5 to 29.3 per cent, and were significantly greater than the average mortality of the unselected mice, except in the cases of Lines 4, K, L, and M.

The second selection was made (Table III) by discarding all but five of the eighteen lines of the first selection, together with individuals in the five selected lines which by the progeny test were least susceptible. From the five selected lines further second generation progeny were obtained (S_2) and mated brother to sister. Their offspring were

TABLE III
Susceptible Lines of Rockefeller Institute Mice. Results of Progeny Tests Following Second and Third Selections

Second selection							Third selection						
Line identification	No. sires	No. dams	Progeny tested				Line identification	No. sires	No. dams	Progeny tested			
			No. litters	No. progeny	No. dead	Dead per cent				No. litters	No. progeny	No. dead	Dead per cent
1	3	10	18	72	60	83.3	1	11	34	65	295	258	87.5
2	2	7	22	97	81	83.5	2	4	18	31	167	146	87.4
3	2	7	24	124	96	77.4	3	4	17	15	82	65	79.3
4	3	8	20	95	74	77.9	4	3	12	9	50	24	48.0
3 x 4	1	4	13	58	40	68.9	3 x 4	2	7	7	29	24	82.7
5	5	25	45	271	125	46.1	5	1	3	3	20	14	70.0
1 x 5	1	2	3	20	8	40.0	1 x 2	3	9	9	47	34	72.3
Totals...	17	63	145	737	484	65.5		29	104	143	720	593	82.3

then tested, two litters from each dam as stated above. The average mortality of progeny of all lines, 145 litters, totaling 737 mice, was 65.7 per cent as compared to 60.9 in the first selection. This difference is significant. The mortality of progeny of Line 4 was likewise notably higher. However, the mortalities of progeny of lines showing highest mortality on the first test (Lines 1, 2, and 3), together with Line 5, did not differ significantly from those of the first selection. The susceptible Line 1, with 83.3 per cent mortality, crossed with the less susceptible Line 5, with 46 per cent mortality, gave three litters,

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CONCLUSIONS

1. Balance studies have been made on three dogs before and after adrenalectomy, performed in two stages.

2. It has been shown that the sodium concentration of the blood decreases in adrenalectomized dogs, as is true in patients suffering from Addison's disease and in cats experimentally adrenalectomized.

3. There are also decreases in the chloride and bicarbonate concentrations which together are approximately equivalent to the decrease in sodium.

4. An increase in the potassium concentration of the blood occurs after adrenalectomy, as reported in other studies. There is no obvious correlation of this change with changes in potassium balances.

5. The balance studies show a striking loss of sodium from the body during the development of adrenal insufficiency. This loss of Na results from an increased excretion of sodium in the urine and is not complicated by loss of base as a result of vomiting or diarrhea.

6. Following adrenalectomy, both the total amount of sodium and its concentration in the urine are markedly increased. This increase in concentration of sodium occurs in spite of an augmented urine volume.

7. The behavior of the chloride ion following adrenalectomy parallels that of the sodium ion, but the loss is not equivalent.

8. During the period of accumulation of non-protein nitrogen in the blood, the rate of water excretion by the kidney is even greater than before removal of the adrenal glands.

9. The possibility of a regulatory effect of the adrenal glands upon sodium metabolism and renal function has been discussed.

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per cent; following 50,000 bacilli, 1/100th of the standard dose, 60.4 per cent.

The fifth selection was made by choosing the fourth generation sires and dams whose progeny had proved most susceptible to the small test doses of *B. enteritidis* and obtaining further fifth generation litters, S_5 , for breeding. 19 sires and 48 dams were secured from Line 1 and 16 sires and 39 dams from Line 2. Brother to sister matings were made as usual. 270 progeny, 54 litters, from Line 1, and 197 progeny, 41 litters, from Line 2, were given a test injection of 100,000 *B. enteritidis*, 1/50th of the usual standard dose. The mortality of all progeny, 76.6 per cent, did not differ significantly from that of progeny of the

TABLE V

Susceptible Lines of Rockefeller Institute Mice. Results of Progeny Tests Following Fifth and Sixth Selections

Line identification	No. sires	No. dams	Progeny tested				Remarks
			No. litters	No. progeny	No. dead	Dead <i>per cent</i>	
1	19	48	54	270	215	79.6	Fifth selection, 100,000 organisms per mouse
2	16	39	41	197	143	72.6	
1	6	12	12	74	61	82.4	Sixth selection, 100,000 organisms per mouse

fourth selection, 82.2 per cent, receiving a like dose of *B. enteritidis* (Table V). In Line 1 there was no change, but in Line 2 there occurred a decrease in mortality. This inconsistency is discussed later.

The sixth selection was carried out in a similar manner. At present progeny tests are complete on six sires and twelve dams, the sixth generation (S_6) of Line 1. The mortality of twelve seventh generation litters, totaling 74 mice, 82.4 per cent, following the test injection of 100,000 bacilli (Table V), was not significantly different from that of the sixth generation progeny, 79.6 per cent.

Resistant Lines (Table VI).—The first selection was made by choosing survivors of 600 unselected mice, given *B. enteritidis*, whose litters in turn likewise survived injection. The chosen animals and their subsequent progeny were free of the infection, as proved by re-

INHERITED AND ACQUIRED FACTORS IN RESISTANCE TO INFECTION

I. DEVELOPMENT OF RESISTANT AND SUSCEPTIBLE LINES OF MICE THROUGH SELECTIVE BREEDING*

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(From the Laboratories of The Rockefeller Institute for Medical Research)

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Resistance of the host to infection, although generally regarded as the resultant of both inherited and acquired forces, has been studied most intensively from the viewpoint of the acquired elements which develop from contact of host and microorganism. Recently, however, the less defined influences associated with heredity and environment have been made subject to experimental study.

Geneticists have shown that controlled lines of plants have definite and different amounts of resistance which are inherited. They have observed (1, 2) that intercurrent infections may be more prevalent in some lines of animals than in others housed under similar conditions. They have injected large doses of specific bacteria parenterally into birds and rodents and by breeding from survivors have developed lines increasingly resistant to these injections (1924 to 1933) (3-8). It is not clear, however, that these test animals have been free from the infections studied and from specific immunity factors which could conceivably have developed and invalidated the findings completely. Moreover, it has not been shown that the observed differences in reaction of the animals to the artificial injections are related to differences in resistance to the natural infections.

In 1922 there were developed in this laboratory inbred lines of mice suitable for investigation in that they were proved to have had no exposure to the infections under study and to possess no acquired immunity. Moreover, a technique of infection was developed which was controlled and yet reproduced closely all phenomena of the natural disease. Some lines of the mice proved consistently more and some less susceptible to experimental *B. aertrycke* (9) and *B. enteritidis*

* The author is indebted to Dr. M. R. Irwin for procuring special strains of mice, for formulating a modified Steenbock ration, and for assistance in developing the breeding stock, to Mrs. K. B. Liedke and Miss A. Johnson for assistance in carrying on the work, and to Drs. C. Lynch and J. Gowen for helpful criticisms of results.

FACTORS IN RESISTANCE TO INFECTION. I

TABLE VII—*Concluded*

Stocks compared	Mortality	Significance of differences	
		χ^2	P
Susceptible Line 4 S ₁	<i>per cent</i> 46.7	3.7	0.06
with U.....	37.4		
Susceptible Line 4 S ₂	77.9	21.5	<0.01
with S ₁	46.7		
Susceptible Line 4 S ₃	48.0	13.4	<0.01
with S ₂	77.9		
Susceptible Line 5 S ₁	50.0	7.08	<0.01
with U.....	37.4		
Susceptible Line 5 S ₂	46.2	0.49	0.50
with S ₁	50.0		
Susceptible Line 5 S ₃	60.0	4.36	0.03
with S ₂	46.0		
Resistant all lines R ₁	20.1	15.42	<0.01
with U.....	37.4		
Resistant all lines R ₂	11.3	7.42	<0.01
with R ₁	20.1		
Resistant all lines R ₄	17.5	1.80	0.18
with R ₃	14.8		
Resistant Line 1 R ₁	16.0	13.64	<0.01
with U.....	37.4		
Resistant Line 1 R ₂	9.1	3.30	0.07
with R ₁	16.0		
Resistant Line 1 R ₄	17.8	1.12	0.25
with R ₃	15.6		
Resistant Line 2 R ₁	32.3	0.31	0.52
with U.....	37.4		
Resistant Line 2 R ₂	20.0	2.07	0.15
with R ₁	32.3		
Resistant Line 2 R ₄	15.5	1.29	0.22
with R ₃	10.1		
with R ₁	32.3	4.28	0.04
with U.....	37.4		
		15.92	<0.01

Lexion ratio formula. In Table VIII the results on susceptible Lines 1 and 2 and resistant Lines 1 A, 1 B, and 1 C are given. Litters in susceptible Line 2 did not tend, and litters in susceptible Line 1 and

their entire services to the colony. Temperature is thermostatically controlled at 72°. Floor and walls are washed and wiped weekly with 5 per cent lysol. Racks with five shelves hold the standardized, galvanized iron cages. Bedding consists of autoclaved pine shavings. Cages are cleaned weekly by soaking in 5 per cent lysol and scrubbing with soap and water. These measures have proved successful in keeping out intercurrent infections. The diet, put into clay cups every 2nd day, consists of a modified Steenbock formula suggested by M. R. Irwin—yellow cornmeal, 64 per cent; linseed oil meal, 16 per cent; crude casein, 5 per cent; ground alfalfa, 2 per cent; powdered milk, 5 per cent; wheat germ, 10 per cent; yeast, 2 per cent; sodium chloride, 0.5 per cent; calcium carbonate, 0.5 per cent; cod liver oil, 2 per cent; water is available in drop bottles. All mice are identified by ear clip-pings. Matings are made when mice are 3 months old, one male to four females. Toward the end of the gestation period, the pregnant dams are removed to fresh individual boxes. The number of young born is recorded. When they are 4 weeks old they are separated according to sex and assembled six to eight per box. The dam is returned to the proper breeding box. Weight of young at 4 and 8 weeks is recorded.

Methods of Experimental Infection.—Mice are given the test bacteria when 2 to 3 months old. An 18 hour broth culture of *B. enteritidis* is diluted in broth to contain 5,000,000 organisms per 0.5 cc. This volume of diluted culture is administered by silver catheter intrastomachally to each individual. The animals are then placed in individual glass battery jars containing shavings, food cups, and water, and kept in a special room. This procedure incites an infection which simulates the spontaneous one epidemiologically, clinically, and pathologically.

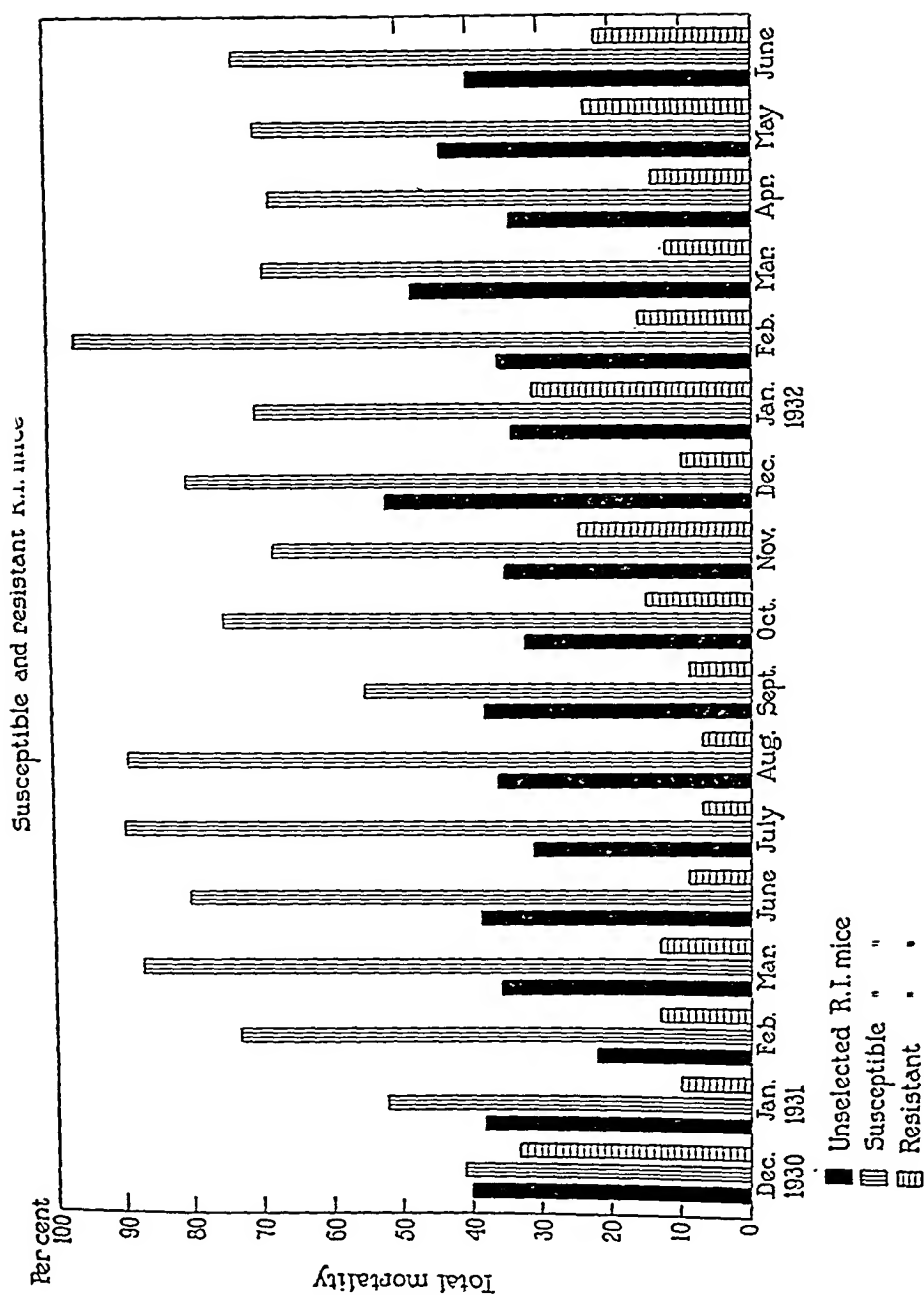
Technique of Selective Breeding Experiments.—The selective breeding of R. I. mice was carried out in the following manner. 500 females were mated at random with 100 males. The young were weaned at 4 weeks. At this time the parents were given the standard dose of *B. enteritidis*. In instances in which both parents died within 10 days after infection, the progeny were reserved as the susceptible stock. Subsequent matings were made, brother to sister. In instances in which both parents survived the infection, the progeny were injected. If all progeny survived, the surviving parents were tested twelve times for the presence of *B. enteritidis* in their stools. Those persistently negative were mated again. In instances in which two tested litters survived, a third litter was obtained and reserved as the resistant stock. Matings were then made brother to sister. *Subsequently, the established breeding stock and all progeny have consisted of animals never exposed to infection.* Further selections have been made entirely on the basis of the progeny test; that is, of per cent mortality of two litters and fertility of sire and dam. In the case of the susceptible lines, animals were discarded if two litters showed a mortality less than 80 per cent, and in the case of the resistant lines, a mortality greater than 20 per cent. The tests were made at the first of each month. At the same time 50 unselected mice, raised under identical conditions, were likewise tested as controls. The same mouse strain of *B. enteritidis* (10) has been employed throughout,

FACTORS IN RESISTANCE TO INFECTION. I

TABLE VII—*Concluded*

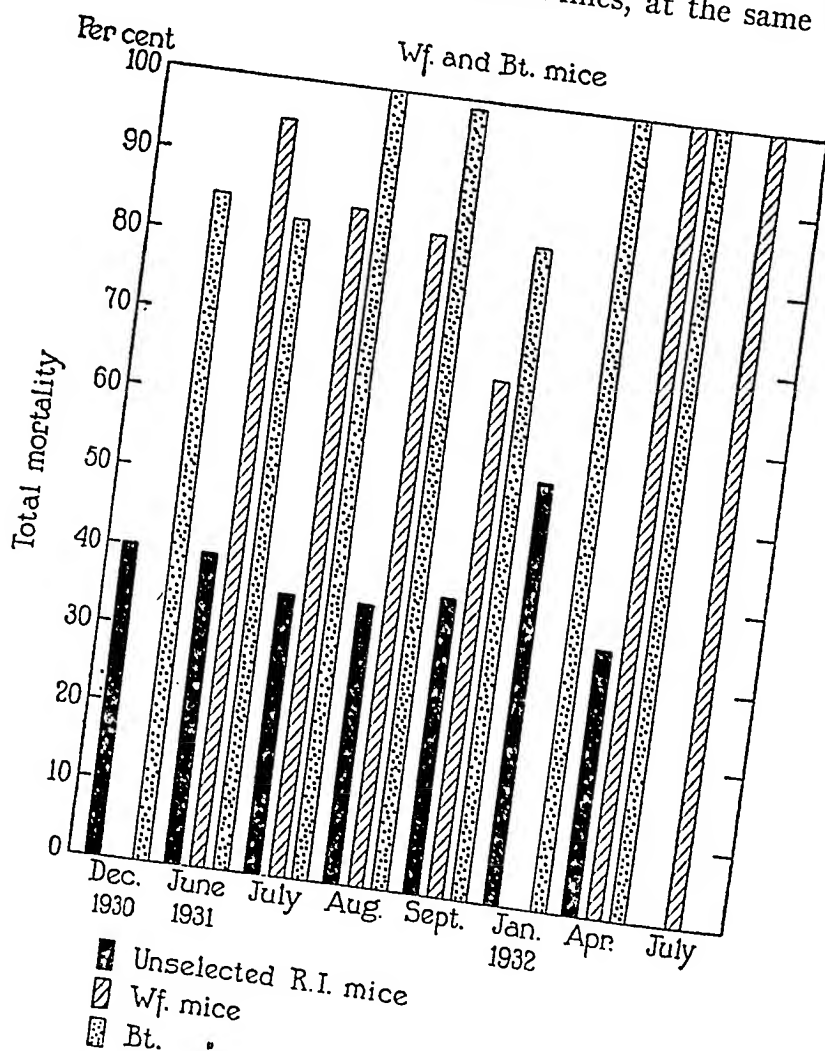
Stocks compared	Mortality	Significance of differences	
		χ^2	P
Susceptible Line 4 S ₁	<i>per cent</i> 46.7		
with U.....	37.4	3.7	0.06
Susceptible Line 4 S ₂	77.9		
with S ₁	46.7	21.5	<0.01
Susceptible Line 4 S ₃	48.0		
with S ₂	77.9	13.4	<0.01
Susceptible Line 5 S ₁			
with U.....	50.0		
Susceptible Line 5 S ₂	37.4	7.08	<0.01
with S ₁	46.2		
Susceptible Line 5 S ₃	50.0	0.49	0.50
with S ₂	60.0		
	46.0	4.36	0.03
Resistant all lines R ₁			
with U.....	20.1		
Resistant all lines R ₂	37.4	15.42	<0.01
with R ₁	11.3		
Resistant all lines R ₄	20.1	7.42	<0.01
with R ₃	17.5		
	14.8	1.80	0.18
Resistant Line 1 R ₁			
with U.....	16.0		
Resistant Line 1 R ₂	37.4	13.64	<0.01
with R ₁	9.1		
Resistant Line 1 R ₄	16.0	3.30	0.07
with R ₃	17.8		
	15.6	1.12	0.25
Resistant Line 2 R ₁			
with U.....	32.3		
Resistant Line 2 R ₂	37.4	0.31	0.52
with R ₁	20.0		
Resistant Line 2 R ₄	32.3	2.07	0.15
with R ₃	15.5		
with R ₁	10.1	1.29	0.22
with U.....	32.3		
	37.4	4.28	0.04
		15.92	<0.01

Lexion ratio formula. In Table VIII the results on susceptible Lines 1 and 2 and resistant Lines 1 A, 1 B, and 1 C are given. Litters in susceptible Line 2 did not tend, and litters in susceptible Line 1 and



TEXT-FIG. 1. Comparative mortalities of unselected and selected R. I. mice following intrastomachal instillation of *B. enteritidis* mouse typhoid bacilli.

Special Strains of Susceptible Mice.—Lines of mice brother to sister inbred for many generations were tested, together with the Rockefeller Institute selected and unselected control lines, at the same time and



TEXT-FIG. 2. Comparative mortalities of unselected R. I. mice and white-face and black-and-tan mice following intrastomachal instillation of *B. enteritidis* mouse typhoid bacilli.

with the same culture. Table IX and Text-fig. 2 show the results of these tests. In Table X the total mortalities of different lines are compared according to the χ^2 test. Rockefeller Institute, hairless,

significant. Further comparisons of the mice are made on the basis of selection.

Susceptible Lines.—The first selection of susceptibles (Table II) was made by choosing the litters of unselected sires and dams, both of which had died 1 to 10 days after injection (see Technique). These

TABLE II

Susceptible Lines of Rockefeller Institute Mice. Results of Progeny Tests Following First Selection

Progeny of Selected Parents Dying 1 to 10 Days after Injection

Line identification	Sire identification	No. dams	Progeny tested			
			No. litters	No. progeny	No. dead	Dead per cent
1	223.0	7	24	136	119	87.5
A	288.0	6	11	52	45	86.5
2	158	4	11	59	43	72.8
3	127	6	20	105	74	70.4
B	3.0	4	12	64	47	73.4
C	277	6	8	43	31	72.0
D	356.6	7	14	57	40	70.1
E	404	6	13	57	40	70.1
F	86.0	4	6	38	26	68.4
G	330.1	6	13	78	47	60.2
H	140	7	23	127	73	57.5
I	81.0	7	13	87	49	56.3
4	106	8	27	120	56	46.7
5	356.0	5	16	122	61	50.0
J	330.2	9	16	98	48	49.0
K	226	5	12	68	32	47.0
L	59	6	14	77	27	35.0
M	256.0	5	7	41	12	29.3
Total.....	18	108	260	1,429	870	60.9

litters, S_1 , or possible lines, eighteen in number, never exposed to infection, were then mated brother to sister and otherwise for progeny tests. From 7 to 27 litters containing 38 to 136 progeny were obtained from each line and tested. The average mortality of the 1,429 progeny from all lines was 60.9 per cent, significantly greater than that of the unselected control mice, 37.4 per cent.¹ The progeny mortalities of

¹ χ^2 tests for significance of differences are given in Table VII.

dwarf, and unselected black-and-tan mice did not differ significantly; likewise, Bagg and pink-eye belonged in the hairless, dwarf, and unselected black-and-tan group. White-face, selected black-and-tan, and spotted strains, however, showed significantly higher mortalities, $P = <0.01$. These differences were consistent in repeated tests.

Cross-Breeding Test

The susceptible white-face strain, relatively homogeneous and stable, with 89 per cent average mortality, was mated with the selected resistant Rockefeller Institute strain, 1 A, relatively stable but not homogeneous, with 15 per cent average mortality. The resulting F_1 progeny were then back-crossed to both susceptibles and resistants and mated together for F_2 progeny. The F_1 , F_2 , and back-crossed progeny were tested simultaneously with a single culture administered according to the standard technique, thus eliminating possible variables associated with the testing of different batches at different times and insuring accurate information of the relative resistance of different lines to the natural infection. The F_2 and back-cross progeny were of standard age—2 to 3 months; the F_1 progeny were 5 to 6 months old, well within the tested age limits of uniform susceptibility.

Dec. 14, 1931. Four males from the selected 85 per cent resistant Line 1 of the R. I. mice were mated each with three or four 90 per cent susceptible white-face line females, and four white-face males each with three or four resistant females. In the first instance, ten F_1 litters, totaling 52 progeny, resulted; in the second, eleven F_1 litters, totaling 72 progeny.

Apr. 2, 1932. Five males from different litters of F_1 (resistant male x susceptible female) and five males from different litters of F_1 (susceptible male x resistant female) progeny were mated back to susceptible and resistant mothers respectively. At the same time, ten F_1 females were back-crossed to two resistant sires and eighteen F_1 females to four susceptible sires. Finally, brother to sister matings were made of the remaining F_1 progeny,—four males to ten females from the (resistant male x susceptible female) group and seven males to nineteen females from the (susceptible male x resistant female) group.

July 1, 1932. All mice were given the standard intrastomachal instillation of 0.5 cc. broth containing 5,000,000 *B. enteritidis* mouse typhoid bacilli. Twenty-eight white-face susceptibles were likewise injected at the same time. The results are given in Table XI.

totaling twenty mice with a mortality of 40 per cent, suggesting dominance of resistance.

In the third selection (Table III), the five lines were retained but less susceptible individuals, as determined by the testing of two litters, were discarded (see Technique). Additional litters were secured from the selected susceptibles (S_3) and brother to sister matings made. The mortality of all progeny from the third selection lines was greater (Table III), 82.3 per cent, than that of progeny from the second selection lines, 65.5 per cent. The third selection increased the mortalities of Lines 4 and 5 significantly but had no appreciable effect on Lines 1, 2, and 3.

TABLE IV

Susceptible Lines of Rockefeller Institute Mice. Results of Progeny Tests Following Fourth Selection

Line identification	No. sires	No. dams	Progeny tested				
			Dose	No. litters	No. mice	No. dead	Dead
							<i>per cent</i>
1	16	76	5×10^6	10	60	51	85.0
			5×10^5	9	38	34	89.5
			10^5	24	129	96	74.4
			5×10^4	9	43	26	60.4
			5×10^3	1	6	6	100.0
2	8	35	10^5	24	124	112	90.3
			10^3	3	18	16	88.8
1 x 2	1	5					
Totals....	25	116		80	418	341	81.5

In the fourth selection (Table IV), all but Lines 1 and 2 were discarded. Further elimination of less resistant and sterile individuals resulted in a choice of 16 fourth generation sires and 76 dams of Line 1 and 8 sires and 35 dams of Line 2, S_4 . Brother to sister matings were made, and from Line 1, 52 litters, totaling 270 progeny, were obtained and tested, and from Line 2, 25 litters, totaling 130 progeny. The dosage of *B. enteritidis* was decreased in order to make the basis for selection more rigid. The mortality following the standard instillation of 5,000,000 bacilli was 85.0 per cent; following 500,000 bacilli, 1/10th the standard dose, 89.5 per cent; following 100,000 bacilli, 74.4

61.2 per cent, is significant. The differences in mortality between the three groups comprising this set are not significant. Finally, of 192 progeny of F_2 generation, 34.2 per cent died. Analysis of mortalities in F_1 , F_2 , and back-cross progeny according to color marking showed no consistent association between the white-face color pattern and susceptibility. Comparisons of sex mortalities in each of the ten sets give no evidence of sex linkage. Taken together the results of this experiment are evidence that definite amounts of resistance to natural *B. enteritidis* infection are inherited, dominant, and not color nor sex-linked.

A final and necessary test of the relative resistance of these lines of mice was made by exposing them to a herd infection.

Experiment 1.—Oct. 15, 1931. Twenty unselected control, R. I. mice were each given an intrastomachal instillation of 5,000,000 *B. enteritidis* bacilli. They were then placed in four cages, five mice per cage. 24 hours later they were changed to fresh cages and to each cage of five injected mice, five selected susceptible and five selected resistants of the R. I. line were added as healthy contacts. Bedding was changed weekly thereafter. Postmortem studies were made on dead mice unless destroyed by cannibalism. Since infections other than *B. enteritidis* typhoid were not encountered, total mortality figures are used to represent mouse typhoid mortality. The results at 8 weeks are given in Table XII. In each of the four communities the mortality of the susceptible contacts surpassed that of the resistant contacts. 30 per cent of the twenty susceptibles died, as compared to 0 per cent of the resistant contacts.

Experiment 2.—Nov. 9, 1931. Twenty selected susceptible R. I. mice were each given the standard instillation of *B. enteritidis* and placed in four cages of five mice each. 24 hours later five susceptible and five resistant R. I. mice were added to each cage as contacts. Further procedures were similar to those in Experiment 1. In each community the mortality of susceptible was greater than that of resistant contacts; the total mortality of the former was 65 per cent as compared to 15 per cent of the latter (Table XII).

Experiment 3.—Jan. 12, 1932. Mice were assembled and treated as in Experiment 2 (Table XII). In each cage the mortality of susceptible contacts exceeded that of resistant contacts; of the susceptibles, 85 per cent, and of the resistants, 20 per cent died.

Experiment 4.—Feb. 2, 1932. Mice were assembled in ten cages, the first six containing five susceptible and five resistant R. I. mice as contacts, Cages 7 and 8, white-face susceptible and resistant R. I. mice, and Cages 9 and 10, black-and-tan susceptible and resistant R. I. mice as contacts (Table XII). In two cages, no mortality occurred among the contacts; in one cage the mortality of susceptible contacts equalled, and in seven cages surpassed that of the resistant contacts. 38 per cent of the susceptible and 8 per cent of the resistant contacts succumbed.

peated tests (see Technique). Three sires and twenty-four dams were chosen (R_1), constituting three possible lines. Matings were made and subsequent litters tested. The mortality of the total 138 tested was significantly less, 20.1 per cent, than that of the unselected mice, 37.4 per cent. The mortality of Lines 1 and 3 proved significantly less, 16

TABLE VI
Resistant Lines of Rockefeller Institute Mice. Results of Progeny Tests Following First, Second, and Third Selections

Line identification	Sire identification	No. sires	No. dams	Progeny tested				Remarks
				No. litters	No. progeny	No. dead	Dead per cent	
1	87	1	8	18	75	12	16.0	First selection, 5,000,000 organisms per mouse
2	71	1	12	7	34	11	32.35	
3	8	1	4	6	30	5	16.6	
Totals....		3	24	31	139	28	20.1	
1		10	42	86	460	42	9.1	Second selection, 5,000,000 organisms per mouse
2		4	14	16	80	16	20.0	
3		1	3	1	6	4	66.6	
Totals....		15	59	103	546	62	11.3	
1		32	134	142	833	130	15.6	Third selection, 50,000,000 organisms per mouse
2		4	13	19	119	12	10.1	
Totals....		36	147	161	952	142	14.8	
1		23	88	90	511	91	17.8	Fourth selection, 50,000,000 organisms per mouse
2		4	15	20	84	13	15.5	
Totals....		27	103	110	595	104	17.5	

per cent, than that of the unselected mice; that of Line 2 did not differ markedly, 32.35 per cent.

The second selection was made within these lines by saving subsequent uninjected litters (R_2) from the parents whose previous progeny had survived the tests. Brother to sister matings were then made and progeny tested. The mortality of the total 546 proved significantly less, 11.3 per cent, than that of the progeny of the first

TABLE XII—*Concluded*

Experiment No.	Cage No.	Mice injected		Susceptible contacts		Resistant contacts	
		No.	Dead	No.	Dead	No.	Dead
5—concluded	5	5	100	5	100	5	0
				4	100		
	6	5	100	3	33	—	—
				5	100	3	0
				4	100	5	0
	7	5	100	3	66	—	—
				5	100	3	66
				4	100	5	20
				3	100	3	33
				194	70	166	12

Experiment 5.—Apr. 13, 1932. A similar test was run with Cages 1 to 5, made up of susceptible and resistant R. I. contacts, and Cages 6 and 7 of susceptible, white-face, and resistant R. I. contacts. Apr. 28, 1932, four black-and-tan susceptibles were added as healthy contacts to each of the seven cages, and May 17, 1932, three susceptible and three resistant R. I. mice were added to each cage. Mortalities at the end of the experiment, June 30, 1932, are given in Table XII. In one of the fourteen tests, the mortality of susceptible contacts equalled, and in thirteen surpassed that of the resistant contacts. 81 of the total 84 susceptible contacts succumbed, 96 per cent, as contrasted with 8 of the 56 resistant contacts, 14 per cent.

Of the five experiments, three were run in four, one in ten, and one in fourteen simultaneous tests. Of the 194 susceptible contacts, 70 per cent died, as compared to 12 per cent of the resistant contacts, $\chi^2 = 123.0$, $P = < 0.01$. In three of the total thirty-six tests, no susceptible and no resistant contacts died; in two tests, the mortality of the susceptibles equalled that of the resistants; in the remaining thirty-two tests, the death rate of the susceptible surpassed that of the resistant contacts. To summarize, selected mice susceptible to an experimental infection which closely approximates the natural one, were equally susceptible to the infection arising under herd conditions, and conversely, selected mice resistant to the experimental infection were correspondingly resistant to the natural one.

TABLE VIII
Homogeneity of Selected Lines

Line	Selection:	No. of litters	L	χ^2	P
S_1	1	24			
	2	18	1.80	78.0	
	3	65	1.77	23.0	<0.01
	4	129	1.82	193.1	0.02
	5	54	1.26	43.4	<0.01
S_2			1.03	58.3	0.02
	1	11			0.65
	2	22	1.53	11.80	
	3	31	1.34	39.60	0.02
	4	124	1.16	43.20	<0.01
White-face	5	41	1.20	39.42	0.17
	1-4		1.33	65.86	0.05
Black-and-tan		54			<0.01
	1-4		1.14	70.74	0.12
		31	1.06	35.03	0.20
R_1A 87♂ x 133♀	1-5				
	1	138			
	2	2	1.28	227.7	
	3	14	—	—	<0.01
	4	59	0.87	10.7	*
R_1B ♂ x 35♀	5	54	1.34	86.7	0.70
		9	1.31	93.9	<0.01
			1.31	15.6	<0.01
	4				0.05
	3	14			
R_1C x 97♀	2	32	0.95	12.7	
		15	1.14	41.6	0.40
			0.86	11.2	0.22
	1	3			0.70
	2	12	—	—	*
nt mortality.	3	19	1.17	16.68	0.10
	4	24	1.08	22.4	0.20
			1.15	32.1	0.10

Line 1 A did tend toward uniformity on repeated selection. Lines, resistant 1 B and 1 C, and susceptible white-face and black-and-tan, were relatively homogeneous at the first selection and continued so.

White-face and black-and-tan strains are described in the next

detrimental to a study of their resistance to natural infection. A technique of measuring the resistance of mice to natural *B. enteritidis* mouse typhoid infection has been developed.

2. The Rockefeller Institute albino strain showed an average 37.4 per cent mortality to *B. enteritidis* mouse typhoid infection.

3. By breeding from progeny of mice dying early after exposure, lines approximately 85 per cent susceptible were obtained; by breeding from unexposed progeny of mice surviving infection, lines 15 per cent susceptible were obtained. Further selection within the most susceptible and most resistant lines did not alter their reaction; selection within the less susceptible and less resistant lines increased the sought factor. These rates remained approximately the same when the susceptibles received 1/100th and the resistants ten times the standard dose. The selected mice reacted consistently when exposed to spontaneous herd infection.

4. Several strains of mice, brother to sister inbred for special factors, did not differ materially in susceptibility. White-face and selected black-and-tan strains, however, proved 89.0 and 97 per cent susceptible.

5. White-face susceptibles mated with resistant Rockefeller Institute mice gave F_1 progeny, 17.7 per cent susceptible. F_1 mice back-crossed to susceptibles gave progeny 61.2 per cent susceptible and back-crossed with resistants gave progeny 26.3 per cent susceptible. F_2 mice mated *inter se* gave progeny 34.2 per cent susceptible. The results indicate that resistance factors are dominant and not sex-linked.

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TABLE VIII
Homogeneity of Selected Lines

Line	Selection ¹	No. of litters	<i>L</i>	χ^2	<i>P</i>
<i>S</i> ₁	1	24	1.80	78.0	
	2	18	1.77	23.0	<0.01
	3	65	1.82	193.1	0.02
	4	129	1.26	43.4	<0.01
	5	54	1.03	58.3	0.02
<i>S</i> ₂	1	11	1.53	11.80	0.65
	2	22	1.34	39.60	0.02
	3	31	1.16	43.20	<0.01
	4	124	1.20	39.42	0.17
	5	41	1.33	65.86	0.05
White-face	1-4	54	1.14	70.74	<0.01
Black-and-tan	1-4	31	1.06	35.03	0.12
<i>R</i> ₁ A 87♂ x 133♀	1-5	138	1.28	227.7	0.20
<i>R</i> ₁ B 87♂ x 35♀	1	2	—	—	<0.01
	2	14	0.87	10.7	*
	3	59	1.34	86.7	0.70
	4	54	1.31	93.9	<0.01
	5	9	1.31	15.6	<0.01
<i>R</i> ₁ C 87♂ x 97♀	4	14	0.95	12.7	0.05
	3	32	1.14	41.6	0.40
	2	15	0.86	11.2	0.22
	1	12	—	—	0.70
	2	19	1.17	16.68	*
	3	24	1.08	22.4	0.10
	4	—	1.15	32.1	0.20
					0.10

* 0 per cent mortality.

resistant Line 1 A did tend toward uniformity on repeated selection. The remaining lines, resistant 1 B and 1 C, and susceptible white-face and black-and-tan,² were relatively homogeneous at the first selection and have continued so.

² The susceptible white-face and black-and-tan strains are described in the next paragraph.

TABLE I
Relation of Susceptibility to Fertility and Body Weight

Lines	Source	Enteritidis Mortality	Stabil-ity	Homo-geneity	Sex linkage	Fertility		Weight	
						No. born	No. weaned*	4 wks.	8 to 12 wks.
R. I. susc. 1-2	Pair	per cent						gm.	gm.
Black-and-tan susc.	"	85.0 ±	+	±	0	7.2 ± 0.29	5.8 ± 0.22	19.2 ± 0.19	27.4 ± 0.26
White-face susc.	"	97.0 ±	+	+	0	7.3 ± 0.24	6.3 ± 0.24	17.0 ± 0.21	23.4 ± 0.24
R. I. res. 1-2	"	89.0 ±	+	+	0	5.8 ± 0.15	4.7 ± 0.24	14.1 ± 0.18	19.3 ± 0.22
		15.0 ±	+	±	0	7.1 ± 0.31	6.1 ± 0.26	22.7 ± 0.26	34.9 ± 0.42

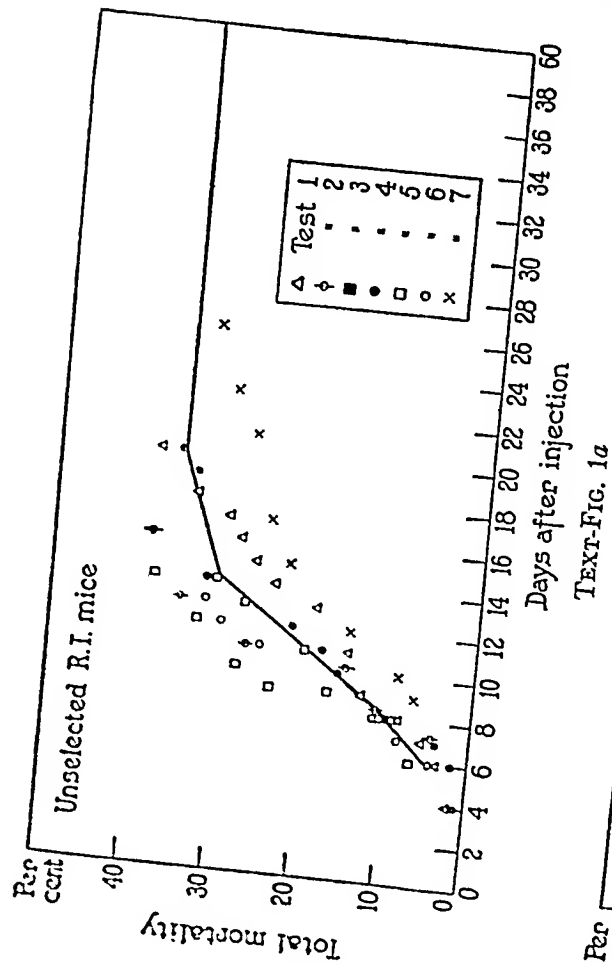
* Number per litter of all large litters reduced arbitrarily to eight.

TABLE IX
Relative Susceptibility of Special Strains of Mice to Intrastomachal Instillation of Enteritidis Mouse Typhoid Bacilli

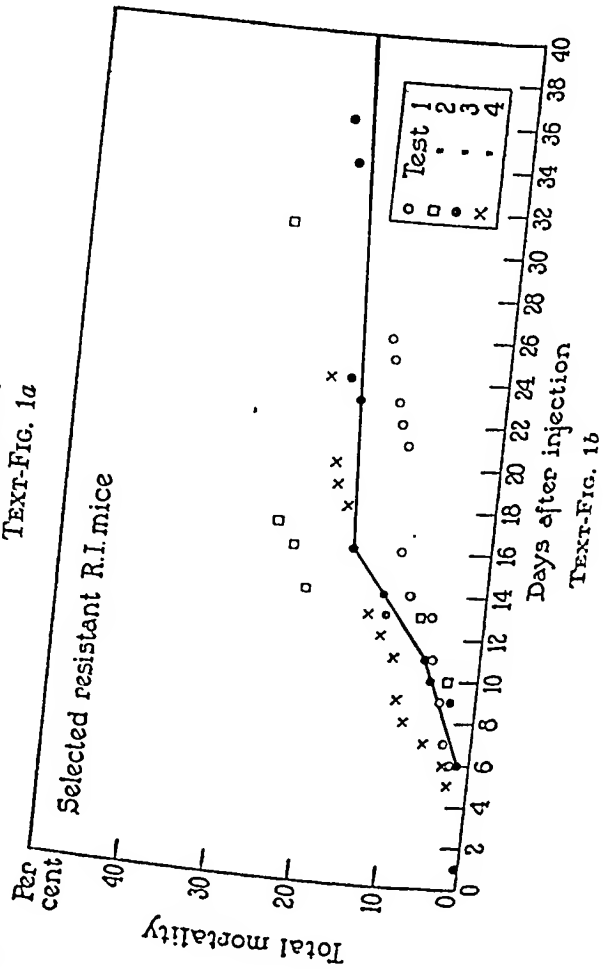
Strain of mice	No. of tests	Total No. tested	Total No. dead	Dead per cent
Bagg.....	8	452	104	23.0
Hairless.....	3	166	52	31.3
Agouti.....	4	69	13	18.8
Pink-eye.....	2	36	7	19.4
Dwarf.....	2	32	9	28.1
Spotted.....	2	8	7	87.5
Black-and-tan unselected.....	6	100	43	43.0
Black-and-tan selected.....	9	117	113	96.5
White-face unselected.....	7	164	146	89.0

TABLE X
Significance of Differences in Mortality of Strains of Mice

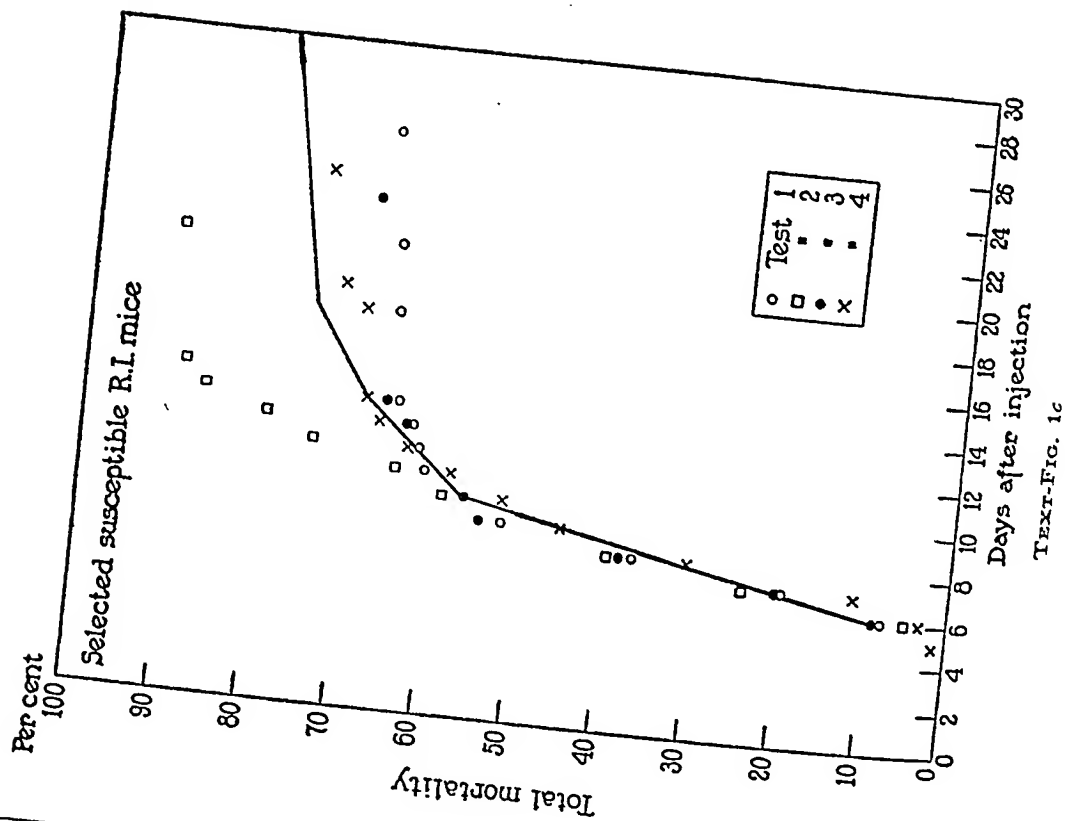
Stocks compared	Mortality per cent	Significance of differences	
		χ^2	P
R. I. unselected.....	37.4	26.5	<0.01
with Bagg.....	23.0	2.1	0.15
" hairless.....	31.0	9.4	<0.01
" agouti.....	19.0	4.8	0.03
" pink-eye.....	27.0	1.1	0.30
" dwarf.....	28.0	8.0	<0.01
" spotted.....	87.5	144.8	<0.01
" white-face.....	89.0	143.0	<0.01
" black-and-tan selected.....	97.0	1.1	0.30
" black-and-tan unselected.....	43.0		
Bagg.....			
with hairless.....	4.31	0.04	
" agouti.....	0.5	0.50	
" pink-eye.....	0.2	0.65	
" dwarf.....	0.4	0.50	
" spotted.....	16.9	<0.01	
" white-face.....	217.2	<0.01	
" black-and-tan selected.....	212.6	<0.01	
" black-and-tan unselected.....	16.5	<0.01	



Text-Fig. 1a



Text-Fig. 1b



Text-Fig. 1c

All the white-face mice died. The original resistant mice were not fit for the test. Of 124 F_1 progeny, 17.7 per cent died, indicating that resistance factors are dominant to those of susceptibility. The differences in mortality between the two groups of F_1 mice are not significant, $\chi^2 = 1.7$, $P = 0.20$. Of 116 progeny from back-cross

TABLE XI
Relative Susceptibility of Progeny of F_1 , F_2 , and Back-Cross Matings of Selected Susceptible and Resistant Mice to Intrastomachal Instillation of *B. enteritidis* Mouse Typhoid Bacilli

Line identification	Parentage	No. litters tested	No. mice tested	No. dead	Dead per cent
F_1 (a)	$R \sigma \sigma \times S \varphi \varphi$	10	52	12	23.0
F_1 (b)	$S \sigma \sigma \times R \varphi \varphi$	11	72	10	13.9
Totals.....		21	124	22	17.7
Back-cross (a)	$F_1 \sigma \sigma (R \sigma \times S \varphi) \times S \varphi \varphi$	7	42	23	54.9
" " (b)	$F_1 \sigma \sigma (S \sigma \times R \varphi) \times S \varphi \varphi$	6	28	26	93.0
" " (c)	$F_1 \varphi \varphi (S \sigma \times R \varphi) \times S \sigma \sigma$	6	46	22	47.9
Totals.....		19	116	71	61.2
Back-cross (a)	$F_1 \sigma \sigma (R \sigma \times S \varphi) \times R \varphi \varphi$	6	39	9	23.1
" " (b)	$F_1 \sigma \sigma (S \sigma \times R \varphi) \times R \varphi \varphi$	1	8	1	12.5
" " (c)	$F_1 \varphi \varphi (R \sigma \times S \varphi) \times R \sigma \sigma$	8	67	20	29.9
Totals.....		15	114	30	26.3
F_2 (a)	$F_1 (R \sigma \times S \varphi)$	7	59	20	34.0
F_2 (b)	$F_1 (S \sigma \times R \varphi)$	19	133	46	34.5
Totals.....		26	192	66	34.2

R = resistant. S = susceptible.

matings of F_1 to susceptibles, 61.2 per cent died. The differences in mortality between the three groups comprising this set are significant and at present must be ascribed to differences in the original material. Of 114 progeny from the back-cross matings of F_1 to resistants, 26.3 per cent died. The difference in mortality between this group, 26.3 per cent, and that from the F_1 to susceptible back-cross matings,

to a total mortality of 30 per cent on the 14th day. After the 21st day, the mortality practically ceased at 37 per cent. Similar consecutive tests on four crude batches totaling 519 selected resistant R. I. mice of Lines 1 and 2 are shown in Text-fig. 1b. The mortality rates were markedly lower, deaths were distributed over a 25 rather than a 10 day period, and the final total mortalities were approximately 50 per cent lower. Tests on batches of selected susceptible R. I., white-face, and black-and-tan mice are shown in Text-figs. 1c, d, e. In these cases the daily mortality rates were about 10 per cent from the 5th to the 12th days. By the 15th day, practically all mice were dead.

Fate of B. enteritidis Following Intrastomachal Infection.—The fact that the susceptible strains succumbed so quickly and in such large numbers, while the similarly exposed resistant strains died less rapidly and in very small numbers, led to an inquiry into the fate of the bacteria after instillation into the stomach to determine whether resistance was localized at the intestinal mucosa portal of entry or was generalized throughout the body.

Experiment 1.—Twenty mice of the selected susceptible and resistant R. I. line and twenty of the unselected Rockefeller Institute mice were each given an intrastomachal instillation of 0.5 cc. of culture containing 5,000,000 *B. enteritidis* mouse typhoid bacilli, and placed in individual glass battery jars. Full details of this procedure have been given elsewhere (1). Blood and stool cultures were taken from each mouse at frequent intervals, dead mice were autopsied and tested for the presence of *B. enteritidis* in blood, spleen, liver, gall bladder, and intestine. Survivors were tested for agglutinins, sacrificed at 50 days, and examined for the presence of *B. enteritidis* in their organs.

The results of this test are summarized in Table II. 70 per cent of the susceptibles died, as compared to 5 per cent of the resistants and 45 per cent of the controls. All mice were discharging *B. enteritidis* in their feces within 8 hours after injection, but the susceptibles continued to be carriers in larger numbers and over longer periods of time than the resistants. Blood cultures from susceptibles contained *B. enteritidis* sooner, more frequently, and in larger numbers and a greater percentage of mice tested than cultures from resistants. A second test was performed in a similar manner, except that blood cultures were taken daily for 11 days, and then every 2nd day until the 24th day. Mortalities in this case were 94 per cent of susceptibles, 15 per cent of resistants, and 50 per cent of controls. The results of blood cultures of both tests are given in Text-fig. 2.

Of the total survivors in both tests, none, with the exception of one susceptible and one control, contained demonstrable agglutinins. 43 per cent of the total seven susceptible survivors and 36 per cent of the thirty-six resistant survivors had shown positive blood cultures for 1 to 10 days; 84 per cent of the former and 15

TABLE XII
Comparative Mortalities of Susceptible and Resistant Strains When Exposed to
Spontaneous Herd Infection (*B. enteritidis* Mouse Typhoid)

Experiments of Susceptible and Resistant Strains When Exposed to Spontaneous Herd Infection (B. enteritidis Mouse Typhoid)							
Experiment No.	Case No.	Mice injected		Susceptible contacts		Resistant contacts	
		No.	Dead per cent	No.	Dead per cent	No.	Dead per cent
1	1	5	20	5	20	5	0
	2	5	0	5	0	5	0
	3	5	40	5	40	5	0
	4	5	60	5	60	5	0
2	1	5	100	5	80	5	0
	2	5	100	5	40	5	0
	3	5	100	5	100	5	0
	4	5	100	5	60	5	20
3	1	5	100	5	80	5	0
	2	5	60	5	100	5	0
	3	5	100	5	60	5	20
	4	5	100	5	100	5	40
4	1	5	80	5	20	5	0
	2	5	80	5	20	5	0
	3	5	80	5	0	5	0
	4	5	80	5	60	5	20
	5	5	100	5	80	5	40
	6	5	100	5	20	5	0
	7	5	80	5	60	5	0
	8	5	100	5	80	5	20
	9	5	100	5	60	5	40
	10	5	40	5	0	5	0
5	1	5	100	5	100	5	0
	2	5	100	5	20	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
6	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
7	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
8	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
9	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
10	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
11	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
12	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
13	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
14	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
15	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
16	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
17	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
18	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
19	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
20	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
21	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
22	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
23	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
24	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
25	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
26	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
27	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
28	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
29	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
30	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
31	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
32	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
33	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
34	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
35	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
36	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
37	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
38	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
39	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
40	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
41	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
42	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
43	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
44	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
45	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
46	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
47	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
48	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
49	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
50	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
51	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
52	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
53	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
54	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
55	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
56	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0
57	1	5	100	5	100	5	0
	2	5	100	5	100	5	0
	3	5	100	5	100	5	0
	4	5	100	5	100	5	0

TABLE II

Reaction of Selected Susceptible and Resistant and Unselected Control Rockefeller Institute Mice to Intrastomachal Instillation of 5,000,000 *B. enteritidis* Mouse Typhoid Bacilli

Test 1

Mouse No.	Days after instillation										Survivors	
	0	1	2	3	4	7	10	14	17	22	30	Spleen culture at autopsy
S 1	0/0	0/0	0/0	0/0	0/0	+/+	/+	0/+	+ / 0	0/+	0/+	+
2	0/+	0/+	0/+	0/+	+ / 0	D.						
3	0/+	0/+	0/+	0/+	+ / 0	"						
4	0/0	0/0	0/0	0/0	+/+	"						
5	0/0	0/0	0/0	0/0	+/+	+/+	D.					
6	0/0	0/0	0/0	0/0	+/+	+/+	+/+	0/+	0/+	0/+	0/+	+
7	0/+	0/+	0/0	0/0	0/0	+/+	+/+	0/+	0/+	0/+	0/+	+
8	0/+	0/+	0/0	0/0	+/+	+/+	D.					
9	0/+	0/+	0/+	0/+	+/+	+/+	+/+	0/+	0/+	0/+	0/+	+
10	0/+	0/+	0/+	0/+	+/+	+/+	+/+	0/+	0/+	0/+	0/+	+
11	0/0	0/+	0/+	0/+	0/+	+/+	+/+	0/+	0/+	0/+	0/+	+
12	0/+	0/+	0/-	0/+	0/+	+/+	+/+	0/+	0/+	0/+	0/+	+
13	0/+	0/+	0/+	0/+	0/0	+/	+/+	D.	0/+	0/+	0/+	+
14	0/+	0/+	0/-	0/+	0/+	0/	0/+	0/+	0/+	0/+	0/+	+
15	0/+	0/+	0/+	0/-	0/+	+/+	0/+	0/+	0/+	0/+	0/+	+
16	0/+	0/+	0/+	0/+	0/-	0/+	0/+	0/+	0/+	0/+	0/+	+
17	0/+	0/+	0/+	0/+	0/0	+ / 0	D.	+/+	0/+	0/+	0/+	+
18	0/+	0/0	0/0	0/0	0/0	0/0	0/+	+/+	0/+	0/+	D.	0
19	0/+	0/+	0/+	0/+	0/+	0/-	+/+	0/+	0/+	0/+	0/0	0
20	0/-	0/+	0/+	0/+	0/-	+/+	D.	0/+	0/+	0/+	0/0	0
Total, per cent.....	0	0	0	0	30	75	33.3	12.5	12.5	0	0	83.3+
Blood culture.....	65	85	60	70	50	50	88.9	100	75	75	66.6	
Stool culture.....	0	0	0	0	0	20	55	60	75	70	70	
Dead.....	0	0	0	0	0	20	55	60	75	70	70	

DISCUSSION

Individuals of a random lot of mice in which known environmental variables have been controlled have been found to differ in their response to a uniform exposure to a natural infection. These differences, according to experimental evidence, are associated directly with heredity factors. The evidence as here presented is that progeny of animals dying soon after exposure suffer a higher mortality than progeny of animals surviving exposure. On the same basis of selection, lines with maximum, minimum, and intermediate mortality were obtained, indicating that selection *per se* did not direct the process. Again, inbreeding was carried out in both high-mortality and low-mortality lines and hence could not have exerted a directing influence on the process. The differences in mortality of selected lines are significant and have persisted through subsequent generations and have been measured. Again, lines with high mortality crossed with low-mortality lines yielded low-mortality F_1 mice; F_1 mice back-crossed with low-mortality mice yielded progeny with low mortality, and F_1 mice back-crossed with high-mortality mice yielded progeny with high mortality—demonstrating segregation of susceptibility and resistance factors on the basis of heredity. It becomes necessary, therefore, to regard the response of previously unexposed individuals to the mouse strain of *B. enteritidis* as dependent upon quantitative amounts of inherited resistance. The number of factors involved is not known because the material is not entirely homogeneous; apparently different combinations of multiple factors, one or more of which may be dominant, or partially so, are concerned. The relation of sex, fertility, and weight to inherent resistance is treated elsewhere (14). Whether the resistance is local or general, affected by environmental conditions, as, e.g., diet, and by acquired immunity which follows exposure to infection, and finally, how these inherited and acquired components of resistance together affect the response of the host to infection are matters now under investigation.

CONCLUSIONS

1. A colony of inbred mice has been established free from inter-current infections and from the influence of extraneous variables

the resistance of internal tissues of resistants and susceptibles to subcutaneous, intraperitoneal, and intravenous injections of fixed doses of *B. enteritidis* directly into the body.

Experiment 2.—May 12, 1932. Ten susceptible R. I., ten susceptible white-face, ten susceptible black-and-tan, and ten resistant R. I. mice were each given intraperitoneally 0.5 cc. of an 18 hour culture of *B. enteritidis* diluted to contain 50,000 organisms. Four similar batches of ten mice were each given the same dose subcutaneously into the groin. Duration of life was recorded and mice dying after the injection were autopsied and tested for the presence of *B. enteritidis*. The thirty susceptible mice injected intraperitoneally died between the 3rd and 9th days (Text-fig. 3); the ten resistants receiving like treatment died more slowly. Three survived (30 per cent) and appeared healthy at the end of the 30 day period of observation. Of animals given the subcutaneous injection, all but one of the thirty susceptibles died between the 4th and 12th days. The remaining mouse died on the 29th day (Text-fig. 4). Of the ten resistant mice treated similarly, seven (70 per cent) survived and appeared healthy on the 30th day. The surviving resistants when sacrificed and tested all harbored *B. enteritidis* in their spleens.

Three other similar tests gave like results, indicating that the internal tissues of susceptible mice are less resistant than those of refractory mice.

Experiment 3.—May 4, 1932. Ten mice of each of the susceptible R. I., white-face, black-and-tan, and resistant R. I. lines were each given into the tail vein 0.5 cc. of an 18 hour culture of *B. enteritidis* diluted to contain 50,000 bacilli. Blood cultures were taken from the tail vein daily thereafter, mice dying of the infection were autopsied and tested for the presence of *B. enteritidis*, and survivors, after 42 days, were tested for agglutinins and then sacrificed and cultured for *B. enteritidis*. The results are shown in Table III and Text-fig. 5. 40 to 50 per cent of each batch yielded positive blood cultures 3 to 5 hours after injection. 24 hours later, all but 2 susceptibles and 2 white-face were negative. On the 2nd day, 45 per cent, and on the 3rd day, 81 per cent of the susceptibles gave positive cultures. By the 13th day, all susceptibles were dead. Meanwhile, one resistant developed a positive blood culture on the 5th day which persisted 22 days and then became negative. No other resistants gave positive blood cultures and all survived and were healthy 42 days. None showed agglutinins. When sacrificed and tested for *B. enteritidis*, 6 (60 per cent) gave positive spleen cultures.

A second test gave similar results. Taken together, the evidence has been consistent that the tissues of innately susceptible mice are more susceptible to *B. enteritidis* than those of innately resistant mice.

INHERITED AND ACQUIRED FACTORS IN RESISTANCE TO INFECTION

II. A COMPARISON OF MICE INHERENTLY RESISTANT OR SUSCEPTIBLE TO *BACILLUS ENTERITIDIS* INFECTION WITH RESPECT TO FERTILITY, WEIGHT, AND SUSCEPTIBILITY TO VARIOUS ROUTES AND TYPES OF INFECTION

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In a previous paper (1), selective breeding experiments on mice were described, in which from a stock 37.4 per cent susceptible to *B. enteritidis* mouse typhoid, certain lines were developed approximately 85 and 15 per cent susceptible, respectively. This paper deals with questions of whether this innate resistance to infection is localized at the portal of entry, or is general throughout the body, whether it is a resistance to a single microorganism, or to a number of harmful agents.

The source and maintenance of the test mice have been described elsewhere and emphasis has been laid on the advantage both of employing animals free from intercurrent disease and of testing their resistance to a natural rather than artificial infection. Each line, save susceptible Line 1, was derived from a single pair and has proved fairly stable in its susceptibility to *B. enteritidis*. Susceptible lines, white-face and black-and-tan, and resistant Lines 1 B and 1 C are homogeneous according to the Lexion ratio formula (1); susceptible Lines 1 and 2 are not sufficiently so to meet the requirements of the test. Details of the standardization of test mice, test cultures, and injection techniques have also been presented.

Sex, Fertility, and Body Weight

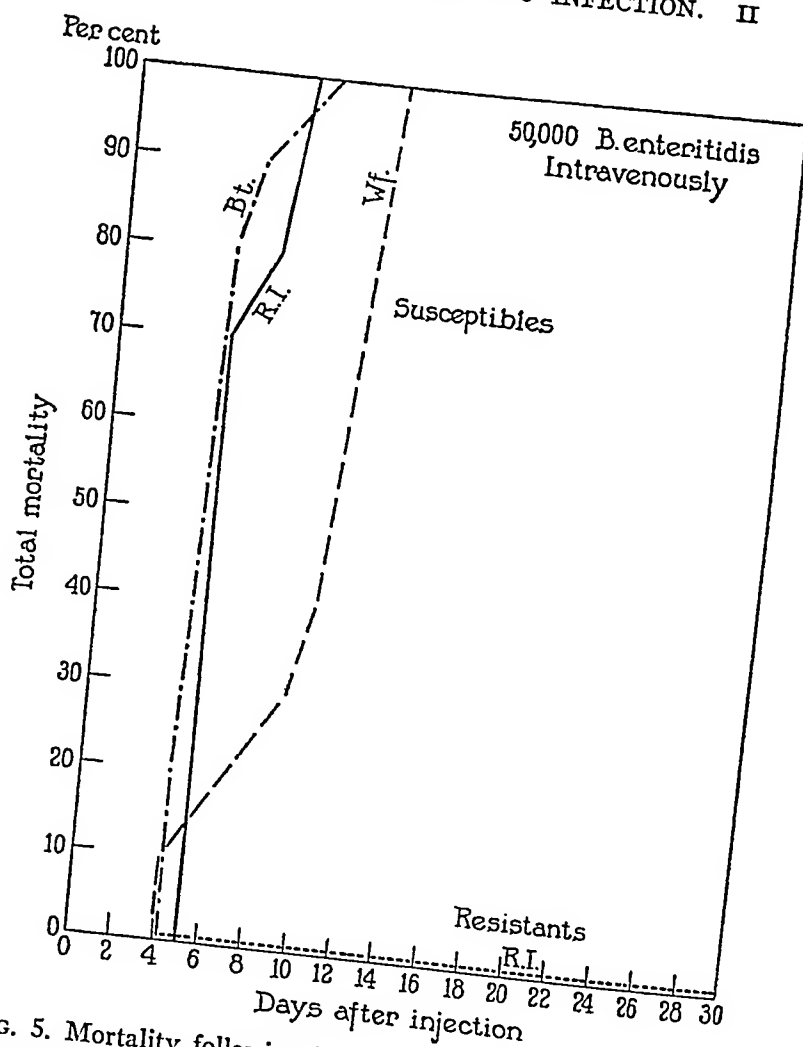
The susceptible and resistant lines were compared with respect to sex ratios, relative fertility, and weight (Table I).

50 consecutive litters of 336 Rockefeller Institute susceptibles of Lines 1 and 2 were compared with 50 consecutive and contemporary litters of 312 R. I. resistants of Lines 1, 49 litters of 283 white-face, and 43 litters of 301 black-and-tan mice. The ratios of number of males to females were similar throughout. Moreover, the comparative mortalities of males and females in tested litters of each line were alike. The mean numbers per litter born in different lines were as follows: susceptible R. I. mice, 7.2 ± 0.29 (standard error of mean), susceptible black-and-tan mice, 7.3 ± 0.24 and resistant R. I. mice 7.1 ± 0.31 . The differences were not significant, $P = 0.9$. The mean numbers per litter born in the susceptible white-face line were 5.8 ± 0.15 , significantly lower than those in the other lines, $P = < 0.01$. The mean numbers per litter weaned, after arbitrarily reducing the number per litter of all large litters to eight, were as follows: susceptible R. I. mice 5.8 ± 0.22 , susceptible black-and-tan mice, 6.3 ± 0.24 , resistant R. I. mice, 6.1 ± 0.26 , and susceptible white-face mice 4.7 ± 0.24 . Again, the differences between resistant and susceptible Rockefeller Institute and black-and-tan lines were not significant, while that between the susceptible white-face lines and others was significant. The mean weights of individuals in these litters at 4 weeks were, —susceptible R. I. mice, 19.2 ± 0.19 gm., resistant R. I. mice, 22.7 ± 0.26 , black-and-tan, 17.0 ± 0.21 , and white-face, 14.1 ± 0.18 . Weights at the time of injection, 8 to 12 weeks after birth, averaged as follows: susceptible R. I. mice, 27.4 ± 0.26 gm., resistant R. I. mice, 34.9 ± 0.42 , black-and-tan, 23.4 ± 0.24 , white-face 19.3 ± 0.22 . The differences between weights of resistant and all susceptible lines were significant in each case, $P = < 0.01$. There was no correlation in the resistant lines between weight and death or survival following the test injection.

These data indicate that the *B. enteritidis*-susceptible lines were not subnormal either in weight or in fertility and that resistant lines, although relatively heavy, were not consistently more fertile. In short, resistance and susceptibility in these mice were not noticeably correlated with presence or lack of bodily vigor.

Susceptibility of Lines to Intrastomachal, Subcutaneous, Intraperitoneal, and Intravenous Infection with B. enteritidis
Time Distribution of Mortality Following Intrastomachal Infection.—In Text-fig. 1 a-c the time distributions of mortality of individuals of unselected and selected susceptible and resistant strains are compared.

Text-fig. 1a shows the results of consecutive tests on seven batches totaling 274 unselected Rockefeller Institute mice. Each mouse was given a fixed dose of *B. enteritidis* mouse typhoid bacilli under standard conditions (1). Deaths commenced on the 4th day, continued at the approximate rate of 3 per cent daily

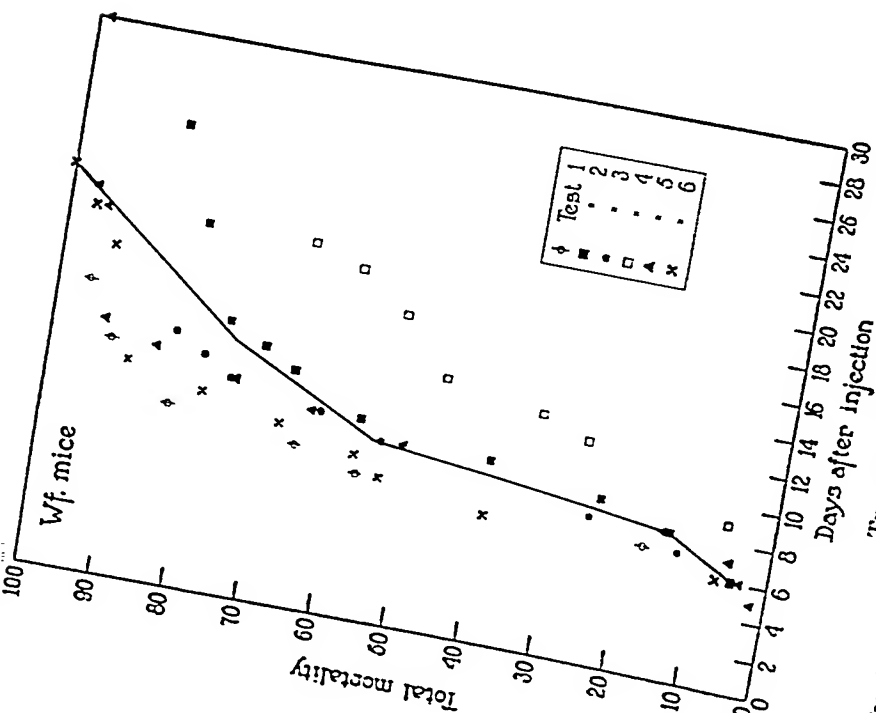


TEXT-FIG. 5. Mortality following intravenous injection of *B. enteritidis* into susceptible and resistant mice.

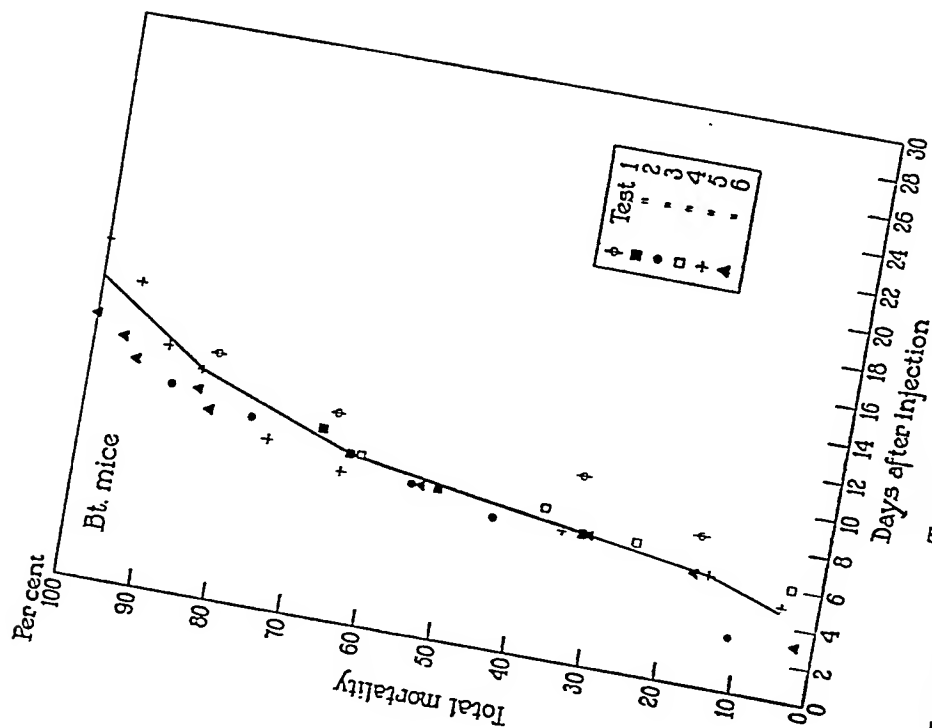
Susceptibility of Lines to Pasteurella, Friedländer, Pneumococcus, and Virus Infections

Knowledge that certain lines of mice were innately susceptible and others resistant to *B. enteritidis* led to a study of whether they were correspondingly susceptible and resistant to other pathogenic agents. *Pasteurella avicida* from a case of fowl cholera, *B. friedlaenderi*, pneumococcus from human cases of pneumonia, and virus associated with louping ill were used as test agents.

Pasteurella avicida—Pasteurellae in rabbits, fowl, and mice give



TEXT-FIG. 1 a-c. Time distribution of mortality following per os instillation of 5,000,000 *B. enteritidis* mouse typhoid bacilli in susceptible and resistant mice.



TEXT-FIG. 1c

TABLE IV
Relative Resistance of *B. enteritidis*-Susceptible and Resistant Mice to Intranasal *Pasteurella avicida* Infection

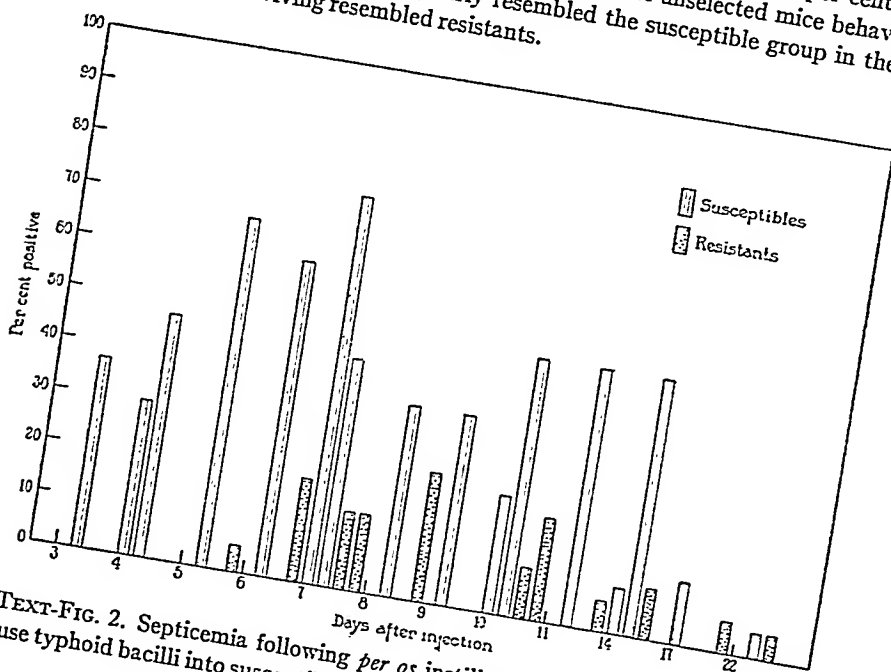
Date	Dose*	Susceptible R. I.			White-face			Black-and-tan			Resistant R. I.			Significance of differences																			
		No. tested	No. died	Mortality	No. tested	No. died	Mortality	No. tested	No. died	Mortality	No. tested	No. died	Mortality	Duplicate tests, susceptibles		Duplicate tests, resistants		Susceptibles and resistants		White-face and resistants		Black-and-tan and resistants		Suscepti- bles and white-face									
														χ^2	P	χ^2	P	χ^2	P	χ^2	P	χ^2	P	χ^2	P	χ^2	P	χ^2	P				
1932																																	
Jan. 6	3	20	13	65	20	12	60	20	7	35	20	7	35	0.10	0.76			4.1	0.05														
Feb. 26	4	20	6	30	20	6	30	20	3	15	20	3	15		Not sig.	1.11	0.24	5.0	0.03														
Mar. 8	5	20	5	25	20	10	50	20	5	25	20	0	0	0.12	0.74			6.7	<0.01	13.3	<0.01			5.7	0.02					2.9	0.10		
May 13	5	20	6	30	20	13	65	20	6	30	20	2	10					3.6	0.05	12.9	0.01			2.5	0.10					3.6	0.05		

* Dose 3 = 30,000 *Past. avicida* per mouse.

" 4 = 3,000 " " "

" 5 = 300 " " "

per cent of the latter were chronic carriers. When sacrificed and tested for the presence of *B. enteritidis* in tissues, 71 per cent of susceptible and 52 per cent of resistants yielded positive spleen cultures. The control unselected mice behaved like a mixed sample,—those dying early resembled the susceptible group in their reactions; those surviving resembled resistants.



TEXT-FIG. 2. Septicemia following *per os* instillation of 5,000,000 *B. enteritidis* mouse typhoid bacilli into susceptible and resistant mice.

The facts that *B. enteritidis* given intrastomachally to susceptibles appeared in the blood stream more promptly, in larger numbers, and in a greater percentage of cases, and was present in feces in larger numbers, for a longer period, and in a greater percentage of cases than when given to resistants indicate that the portal of entry, the intestinal mucosa of susceptibles, was less of a barrier than that of resistants.

The barrier of resistants, however, was not perfect, since 50 per cent of those injected were found to harbor typical *B. enteritidis* in their spleens 50 days later.

Susceptibility of Lines to Subcutaneous, Intraperitoneal, and Intravenous Injections of B. enteritidis.—The entrance and persistence of the organisms in the body of these animals without apparent harm suggest that not only their surface tissues but internal ones as well were relatively resistant. This supposition was tested by comparing

FACTORS IN RESISTANCE TO INFECTION. II

TABLE V
Relative Resistance of B. enteritidis-Susceptible and Resistant Mice to Intranasal Friedlander's Bacillus Infection

TABLE V																													
Acute Resistance of <i>B. enteritidis</i> -Susceptible and Resistant Mice to Intranasal Friedlander's <i>Bacillus</i> Infection																													
Date	Dose*	Susceptible R. I.			White-face			Black-and-tan			Resistant R. I.			Significance of differences															
		No. tested		Mortality	No. tested		Mortality	No. tested		Mortality	No. tested		Mortality	No. tested		Mortality	Duplicate tests, susceptibles		Duplicate tests, resistants		Susceptibles and resistants		White-face and resistants		Black-and-tan and resistants		Susceptibles and white-face		
				per cent			per cent			per cent			per cent			per cent	χ^2	P	χ^2	P	χ^2	P	χ^2	P	χ^2	P	χ^2	P	
1932																													
June 13	4	20	16	80	20	13	65	20	11	55	20	9	45																
Oct. 18	4	20	6	30	20	14	70	20	3	15	20	3	15																
		20	7	35							20	3	15	0.0	Not sig.	5.2	0.02	3.4	0.07	18.0	<0.01	0.20	0.50	0.4	0.50	1.1	0.24	7.5	<0.01
* Dose 4 = 3,000 organisms per mouse.																													

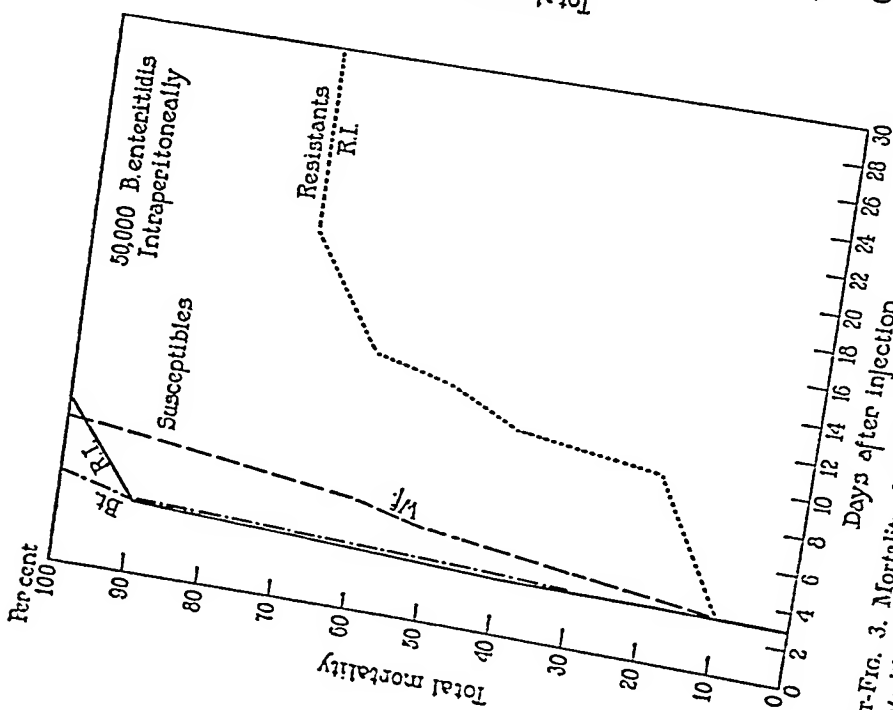
* Dose 4 = 3,000 organisms per mouse.

	65	40	15	15	30	15	35	10	10	20	5	5.3	26.3	68.4
R 41	0/+ +	0/+ +	0/+ +	0/+ +	0/+ +	0/+ +	0/+ +	0/+ +	0/+ +	0/+ +	0/+ +	0/+ +	0/+ +	0/+ +
42	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
43	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0
44	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0
45	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0
46	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0
47	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0
48	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0
49	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0
50	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0
51	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0
52	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0
53	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0
54	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0
55	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0
56	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0
57	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0
58	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0
59	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0
60	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0	0/+ +	0/0
Total, per cent.....														
Blood culture.....														
Stool culture.....														
Dead.....														

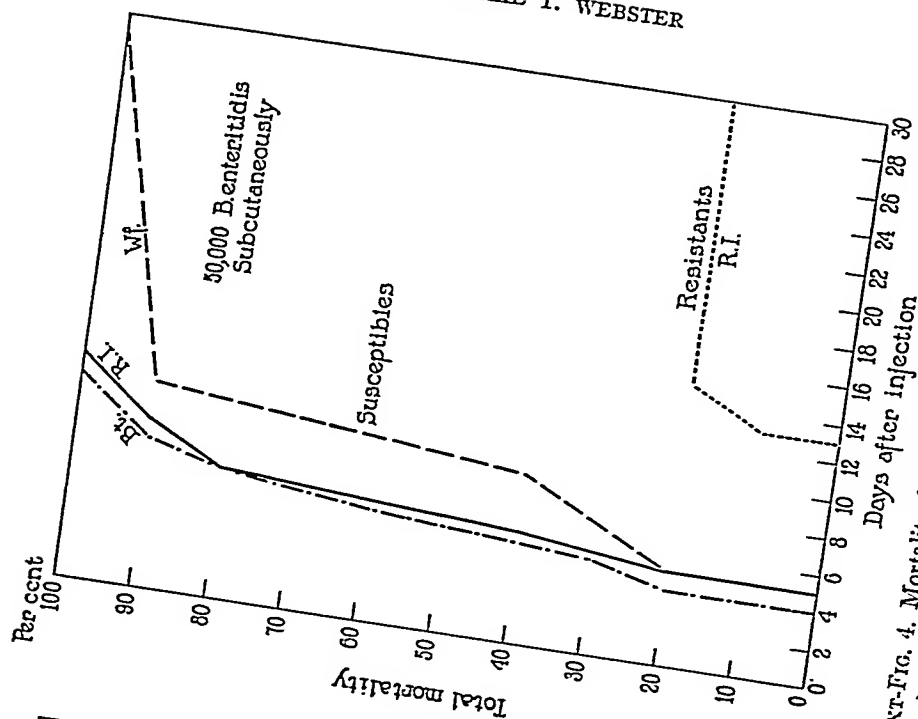
Symbols to the left of diagonal line indicate blood cultures.
 " " "right" " " " stool "

TABLE VI
Relative Resistance of B. enteritidis-Susceptible and Resistant Mice to Intranasal Pneumococcus Infection

Date	Culture No.	Significance of differences											
		Susceptible R. I.			White-face			Black-and-tan			Resistant R. I.		
		No. tested	No. died	Mortality per cent	No. tested	No. died	Mortality per cent	No. tested	No. died	Mortality per cent	No. tested	No. died	Mortality per cent
1931													
Dec. 18	III F	20	7	35							20	5	25
		20	10	50							20	6	30
1932													
Jan. 5	III F	20	8	40									
" 15	III F	20	7	35							20	3	15
		20	9	45							20	1	5
Feb. 4	III F	20	5	25							20	2	10
" 25	III B	20	3	15							20	2	10
		20	4	20							20	1	5
May 17	III C	20	5	25							20	0	0
June 10	VIII	20	9	45							20	0	0
		20	7	35	18	80	20	7	35	20	4	20	0
					10	50	20	2	10	20	0	0	0
		Duplicate tests susceptible			Duplicate tests resistant			Susceptibles and resistant			White-face and resistant		
		χ^2	P		χ^2	P		χ^2	P		χ^2	P	
		0.92	0.36	0.12	0.80			1.9	0.17				
		0.10	0.75	1.1	0.30			8.3	<0.01				
		0.20	0		Not sig.			7.1	<0.01				
		0.70	0		Not sig.			1.1	0.24	10.2	<0.01	0	
								10.1	<0.01		Not sig.	5.56	0.02
								2.8	0.10	20.0	<0.01	1.1	
								8.4	<0.01	13.3	<0.01	2.1	
											0.24	9.22	<0.01
											0.15	0.90	0.30



TEXT-FIG. 3. Mortality following intraperitoneal injection of *B. enteritidis* into susceptible and resistant mice.



TEXT-FIG. 4. Mortality following subcutaneous injection of *B. enteritidis* into susceptible and resistant mice.

TABLE VII
Relative Resistance of B. enteritidis-Susceptible and Resistant Mice to Intranasal Louping Ill Virus Infection

Date	Dilution	Significance of differences											
		Susceptible R. I.			White-face			Black-and-tan			Resistant R. I.		
		No. tested	No. died	per cent mortality	No. tested	No. died	per cent mortality	No. tested	No. died	per cent mortality	No. tested	No. died	per cent mortality
1932													
Dec. 19	1/20	20	8	40	20	20	100				20	12	60
" 27	1/10	15	4	26	20	17	85						
" 29	1/15	15	3	20	16	16	80	20	18	90	25	16	80
		15	5	33	20	16	80	20	16	80	20	11	55
		15	6	40				20	14	70	20	8	40
								20	16	80			
		R. I. susceptibles and resistants			White-face and susceptibles			Black-and-tan and susceptibles			White-face and resistants		
		χ^2	P		χ^2	P		χ^2	P		χ^2	P	
		1.6	0.20		17.1	<0.01					10.0	<0.01	
		25.4	<0.01		22.6	<0.01					0.3	0.60	
		0.7	0.63										
								10.46			<0.01		
											6.3		
											<0.01		

[illegible]

fate of individuals following primary exposure to a natural infection. Innate susceptibility or resistance factors in the genetic sense were not sex-linked or related to body vigor, as expressed by unusual fertility or weight. Indeed, in so far as these experiments are concerned, all lines save possibly the white-face, were sturdy and normal. The genetic factors are probably multiple, with resistance dominant to susceptibility. Again, the tissues of susceptible mice, not only at the surface but throughout the body, appeared more sensitive to *B. enteritidis* than those of resistant mice, suggesting the general rather than local influence of the inherent factors. Finally, the facts that the white-face line proved relatively susceptible to enteric, respiratory tract, and virus infections, the Rockefeller Institute susceptible lines relatively susceptible to enteric and respiratory tract, but resistant to virus infections, the Rockefeller Institute resistant lines resistant to enteric and respiratory and susceptible to virus infections, together with previous observations (8-10), indicate that genetic factors segregated by selective and brother-sister inbreeding and concerned with susceptibility or resistance to infection can operate consistently against a number of, but not necessarily all, harmful agents.

CONCLUSIONS

1. Mice with relatively great inherent resistance to certain bacterial infections were heavier but not more fertile than mice with relatively little inherent resistance. Mice with relatively little inherent resistance were with one exception not abnormally low in weight.

2. *B. enteritidis* given intrastomachally to susceptibles appeared in the blood stream more promptly, in larger numbers, and in a greater percentage of cases, and was present in feces in larger numbers, for a longer period, and in a greater percentage of cases than when given to resistants.

3. Mice relatively resistant to *B. enteritidis* administered by the natural gastrointestinal route were likewise resistant to the organisms introduced subcutaneously, intraperitoneally, and intravenously. Mice relatively susceptible to the organisms administered by the natural route were susceptible when the organisms were injected directly into tissues and blood stream.

rise to an explosive septicemic infection with high mortality. Instilled into the nares, the normal portal of entry of these animals, they set up an infection similar to the natural one (2). Hence in the following tests the mice received the organisms intranasally for the purpose of comparing their resistance to a controlled and at the same time natural infection.

Four tests were made, the first two with two sets of twenty susceptible and twenty resistant R. I. mice run in duplicate, the third and fourth tests with one set of twenty susceptible and twenty resistant R. I. and twenty susceptible white-face and twenty black-and-tan mice. Each animal received intranasally 0.03 cc. of a fixed dilution of an 18 hour rabbit blood culture of *Past. arvicola*, Kansas strain. The doses per mouse in different tests ranged from 15,000 to 150 organisms. The infectivity of this culture in mice has been described elsewhere (2). After receiving the organisms, each animal was placed in a separate battery jar and observed 30 days. Mice dying during this period were autopsied and tested for the presence of the *Pasteurella* strain. The results of the tests are summarized in Table IV.

In Test 1, Table IV, the mortalities of duplicate batches of susceptibles were 65 and 60 per cent, as compared to 35 per cent of resistants. The difference in mortality between the two duplicate susceptible sets is not significant, $\chi^2 = 0.10$, $P = 0.76$; the mortality of susceptibles is, however, significantly greater than that of resistants, $\chi^2 = 4.1$, $P = 0.05$. In Test 2 the mortalities of the duplicate batches of susceptibles were 30 per cent in each case; that of the resistants, 5 and 15 per cent. Again, the differences in mortality between duplicate batches were not significant, while the mortalities of susceptibles were significantly higher than those of resistants, $\chi^2 = 5.0$, $P = 0.03$. Similarly in Tests 3 and 4, the difference in mortalities of duplicate batches was negligible, while the mortalities of susceptibles, white-face, and black-and-tan mice were consistently and with one exception (black-and-tan strain in Test 4) significantly greater than those of resistant R. I. mice. Finally, it appeared that the mortality of the white-face mice was notably greater than that of the R. I. susceptibles. Of a total of 140 susceptible mice, 55 died, 40 per cent; of 40 white-face, 23, 57 per cent; of 40 black-and-tan, 11, 27.5 per cent; and of 100 resistant, 13, 13 per cent died. These differences between susceptibles and white-face on the one hand and the resistants on the other, are highly significant.

It is concluded, therefore, that the Rockefeller Institute susceptible and white-face lines relatively susceptible to a natural *B. enteritidis* mouse typhoid infection were likewise relatively susceptible to a natural *Pasteurella arvicola* infection, and that the Rockefeller Institute resistant line relatively resistant to the enteric was likewise resistant

to the respiratory infection. The black-and-tan line equal to the Rockefeller Institute susceptible and white-face lines in susceptibility to *B. enteritidis* appeared relatively less reactive to *Pasteurella*. *B. friedlaenderi*.—Previous experience (3) has shown that mice given Friedländer-like bacilli by way of the nasal passages develop a fatal infection with pulmonary involvement and septicemia resembling the natural disease.

Mice in batches of twenty were given intranasally 0.03 cc. of an 18 hour broth culture of *B. friedlaenderi* diluted 1 to 10,000, a dose per mouse of about 1,500 organisms. Animals were then placed in individual jars and treated as in the previous experiment. The results of two tests with different strains are given in Table V. Duplicate batches tested showed no significant differences in mortality; white-face and R. I. susceptibles showed consistently, although not always significantly higher mortalities than the R. I. resistants. 29 of the total 60 susceptibles died, 48 per cent; 27 of 40 white-face, 67.5 per cent; in contrast with 15 of 60 resistant, 25 per cent. These differences are significant. Of 40 black-and-tan, 14 or 35 per cent died, a proportion not materially different from that of the R. I. resistants. White-face mice proved again to be more susceptible than the R. I. susceptibles.

The results of these tests are consistent with the previous one in showing that the Rockefeller Institute and white-face strains, relatively susceptible to *B. enteritidis* and *Pasteurella avicida*, are likewise susceptible to *B. friedlaenderi*, and that the Rockefeller Institute strains relatively resistant to the former organisms are resistant to the latter. The tests showed also that the white-face mice are relatively more and the black-and-tan mice less susceptible than the Rockefeller Institute susceptibles to both *Pasteurella avicida* and *B. friedlaenderi*.

Pneumococcus.—Pneumococci from human cases sprayed (4), or instilled (5) into the nares of healthy mice give rise, under certain conditions, to a fatal infection. This infection has been found to be not unlike the natural one in many of its characteristics (6); consequently, it has been utilized in the present studies.

Mice in batches of twenty were given the stated dose of organisms intranasally. From a 15 hour mouse heart blood culture, a 1/100 dilution was prepared in pneumococcus broth and instilled in 0.03 cc. quantities into the nares of each individual, a dose of about 50,000 organisms. The animals were then placed in separate jars, observed for a period of 2 weeks, and when found dead, autopsied and cultured for the presence of the test organism.

shown that the nodules in the two conditions present striking similarities and the hypothesis is advanced that the lesions are manifestations of the same, fundamental, pathological process. A clinical study on the relationship between rheumatic fever and rheumatoid arthritis will form the subject of a succeeding communication.

Gross Appearance and Clinical Characteristics of Subcutaneous Nodules in Rheumatic Fever and Rheumatoid Arthritis

The essential features of the subcutaneous nodules which occur in rheumatic fever are so well known that a detailed description of their gross appearance and clinical characteristics is scarcely necessary. Findlay (8) has recently given a complete description of these lesions. The more important clinical features of the nodular lesions in this disease are tabulated below in comparison with those of rheumatoid arthritis as we have observed them.

Rheumatic Fever

Frequency.—Various authors state that the frequency of nodules in rheumatic fever varies between 10 per cent and 25–50 per cent.

Situation.—In a series of 73 cases Findlay gives the distribution of nodules as follows:

Elbows.....	56
Knees.....	37
Ankles.....	16
Occiput.....	16
Knuckles.....	14
Spine of vertebrae.....	4
Spine of scapulae.....	3

Size.—The nodules are usually about 5 mm. in diameter, but occasionally they

Rheumatoid Arthritis

Frequency.—In the Arthritis Clinic of the Presbyterian Hospital, 245 patients suffering from rheumatoid arthritis have been examined for the presence of nodules. They were found in 66 patients, an incidence of 25 per cent.

Situation.—The nodules in rheumatoid arthritis occur in the same situations as those observed in rheumatic fever, except that they show a relatively greater predilection for the region of the elbows. The distribution in 66 cases was as follows:

Elbows.....	60
Knees.....	8
Ankles.....	6
Occiput.....	2
Knuckles.....	6
Spine of vertebrae (sacral region).....	3

As in rheumatic fever, the nodules may occur in several locations in the same patient and not infrequently present a symmetrical, bilateral distribution.

Size.—As a rule the nodules are considerably larger than those observed in

The results of seven tests are given in Table VI. In the first four tests, a Type III pneumococcus from a fatal case of pneumonia was used. Tests 1, 2, and 3 were run in duplicate by making from the original culture two samples of the stated dilution and giving each to a batch of twenty R. I. susceptibles and twenty R. I. resistants. In each of these tests, the mortalities in duplicate batches were similar; and in the six trials, consistently greater in the susceptible than resistant strains. In the fourth test, white-face and black-and-tan mice were also compared. The mortalities of the susceptible R. I. and white-face batches were greater, while that of the black-and-tan batch equalled that of the resistant batch. In Test 5, duplicate tests with another Type III strain from a fatal case were run with susceptible and resistant R. I. mice. Again, the mortalities in duplicate batches were similar and the mortalities of susceptibles greater than the mortalities of resistant mice. Tests 6 and 7, made with a Type III strain from an apparently healthy human carrier and a Type VIII strain from a case of pneumonia, were run with susceptible and resistant R. I. mice and white-face and black-and-tan mice. In each trial mortalities of R. I. susceptibles, white-face, and black-and-tan mice were greater than those of R. I. resistants. χ^2 tests for significance of differences between mortality of sets showed that duplicate sets gave uniform reactions, that black-and-tan mice did not differ significantly from the resistant mice, but that mortalities of R. I. susceptibles and white-face mice were significantly greater in four of six and in three of three experiments, respectively, than the mortalities of resistants. Finally, mortalities of the white-face mice were significantly greater than those of the R. I. susceptibles in two of three experiments. Of the total 220 R. I. susceptible mice, 74, 33.6 per cent died, and of 60 white-face, 38, 63 per cent died as compared to 24 of 220 R. I. resistants, 11 per cent. These differences are significant. Of 60 black-and-tan, 10, 16.6 per cent died, a proportion not materially different from that of the resistant mice.

In brief, eleven trials with four strains of pneumococcus on four lines of mice showed the susceptible Rockefeller Institute and white-face mice to be more susceptible to pneumococci than the Rockefeller Institute resistant lines, and the white-face more susceptible than the Rockefeller Institute susceptibles. The black-and-tan mice did not differ materially from the Rockefeller Institute resistant mice.

Virus.—The writer and Dr. G. L. Fite have shown louping ill virus obtained from Dr. Rivers to be infectious when introduced into the nasal cavities of certain lines of mice (7). Details of this condition will be reported later. Here it suffices to state that the clinical course, duration of life, gross and histological tissue findings at autopsy rendered the agent suitable for use in these comparative tests.

Microscopic Appearance of Nodules in Rheumatic Fever

The microscopic appearance of the subcutaneous nodules in rheumatic fever has been carefully described by numerous investigators. It is here proposed to review that description only as it bears upon the histogenesis and evolution of the lesion in relation to the nodules which occur in rheumatoid arthritis.

The various stages in the evolution of the rheumatic granuloma as it occurs in various parts of the body, including the myocardium, have recently been carefully described by Klinge (9) and Gross (10). A comparison of the subcutaneous nodule with these lesions reveals a remarkable similarity and it is now recognized by almost all pathologists that the two are "morphologically and genetically essentially the same" (11).

The histological appearance of the rheumatic subcutaneous nodule is in some measure determined by the size of the lesion and the length of time for which it has been present. In this study particular attention has been paid to those nodules in rheumatic fever which are larger in size and which have persisted for some considerable time. Brief reference will also be made to the appearance of the earlier and more acute lesions.

In Fig. 5 there is shown a low power magnification of a rheumatic nodule from a patient 10 years of age who suffered from rheumatic carditis. The nodule had been present over the external malleolus for $2\frac{1}{2}$ months at the time of excision. With the aid of Masson's trichrome stain, which is particularly well adapted to the study of collagen changes, it is seen that the lesion consists of multiple, conglomerate areas of varying sizes and shapes. Each individual area is composed of three more or less well defined zones: an area of central necrosis, a surrounding cellular zone and an enclosing zone of well vascularized, fibrous, connective tissue.

The essential features of these lesions are familiar to all investigators and have been well described by Fahr (12), Swift (13) and numerous others. In its early stages the lesion is composed of a small area of focal necrosis with well marked inflammatory cell infiltration (Fig. 7). Early in the development of the lesion collagen bundles become involved in a peculiar type of fibrinoid swelling and degeneration. It was formerly held that fibrin deposition constituted a prominent feature of the central portion of the nodule but the distribution and staining reactions of the material have led us to prefer Klinge's term "fibrinoid swelling." With hematoxylin-eosin the central area stains more or less uniformly pink, but with Masson's trichrome it presents a fibrillar structure which stains a brilliant red (Fig. 1). Occasional leucocytes are scattered throughout. The margin of the necrotic area is composed of a richly cellular zone, the cells of which show a definite tendency to arrange themselves in radial fashion. The characteristics of these cells have been referred to by numerous observers (11), and it is not proposed at this time to present any detailed description of them (Fig. 13). Mention should

0.02 cc. of a fresh 10 per cent mouse brain and cord emulsion in saline was dropped into the nares of test animals. They were then placed in separate jars and observed over a period of 4 weeks.

The results of three tests are given in Table VII.¹ Each test was run in duplicate with results indicating that a satisfactory control of variables had been achieved. That different dilutions of virus in different tests gave results at times inconsistent is not surprising since there is no known method of measuring the quantity of virus and insuring that in different tests a given dilution contains the desired dosage. In Test 1, the white-face mice were significantly more and the R. I. resistant somewhat more susceptible than the R. I. susceptible mice. In Test 2, the duplicate batches of white-face and R. I. resistant mice were significantly more susceptible than the R. I. susceptibles. In Test 3, the duplicate batches of black-and-tan mice were significantly and the R. I. resistant mice slightly more susceptible than the R. I. susceptibles. In each trial, therefore, the mortality of white-face, black-and-tan, and R. I. resistant mice equalled or exceeded that of the R. I. susceptibles. Of a total 80 white-face, 69 or 85 per cent died; of 40 black-and-tan, 30, 75 per cent; of 100 R. I. resistant, 65, 65 per cent died in contrast with 26 of 80 susceptible mice, 32.5 per cent.

The striking finding in this series of tests was that the Rockefeller Institute susceptible line, relatively susceptible to all previously tested pathogens, was relatively refractory to louping ill, and conversely, the Rockefeller Institute resistant line, relatively resistant to other organisms, was relatively susceptible.

A comparison of the four lines of mice with regard to their relative susceptibility to the various infectious agents tested is shown diagrammatically in Text-fig. 6.

Susceptibility of Lines to Intraperitoneal and Intravenous Injections of Respiratory Tract Pathogens

Pasteurella avicida, *B. friedlaenderi*, and pneumococcus have been injected intraperitoneally and intravenously into susceptible and resistant mice. The organisms, under these conditions, are highly virulent. Hence several preliminary tests were made to determine a dosage which would not be overwhelming in the sense that all mice would succumb abruptly and yet which would kill some of the individuals tested. In no case were these efforts successful. The test dose

¹ *Later Note.*—Further experiments of this sort providing ample confirmation of the observations described have been completed in the period between acceptance of the manuscript and the reading of proof.

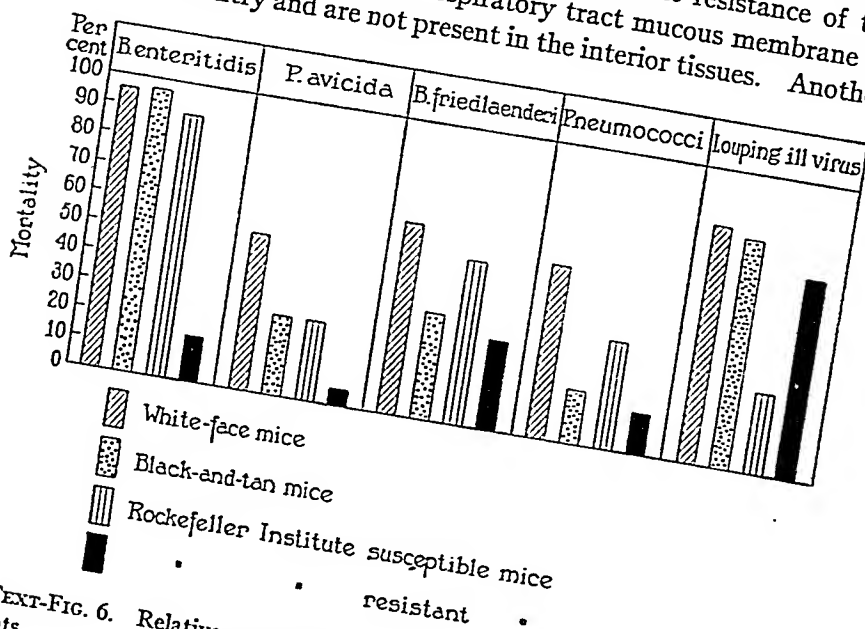
lesions there is a small and not well defined area of focal necrosis characterized by edematous swelling and disintegration of individual collagen bundles. Such early areas of necrosis may show well marked inflammatory cell infiltration chiefly by small round cells and large mononuclear cells although polymorphonuclear leucocytes are not infrequently seen in considerable numbers. The necrotic central areas present the same staining characteristics as those which have been described in rheumatic fever nodules (Fig. 2). The margin of the area of necrosis is again formed by a richly cellular zone, the cells of which are arranged in characteristic radial fashion. The tendency of the cells to be oriented with their long axes perpendicular to the necrotic area contributes to the distinctive appearance of the lesion. In the rheumatoid arthritis nodules this zone appears even richer in cellular elements than in the rheumatic fever nodules and the radial distribution of the cells is more pronounced (Fig. 10). In other respects, however, the cells present an almost identical picture. At this stage of their evolution it is frequently impossible to distinguish the lesions in the two diseases. For the purposes of adequate comparison a more detailed description of the rheumatoid arthritis nodules is here presented.

1. *The Zone of Central Necrosis.*—The appearance of the area of central degeneration in the earlier and smaller lesions has already been described. In the larger and older nodules the character of this area may be considerably altered. The necrotic areas may be extensive and as a rule are irregular in shape. They may be round or irregularly oval, but are more frequently elongated and even branching. For the most part the degenerated area is dense and homogeneous, staining bright to pale pink with eosin, yellow with picric acid in Van Gieson preparations, reddish brown with phosphotungstic acid-hematoxylin, brilliant red in Masson's trichrome and greyish blue or purple in Mallory's orange and aniline blue fuchsin stain.

In other and apparently older nodules, however, the character of the necrotic material has undergone considerable alteration both in structure and staining properties. In some sections the uniform homogeneous character of the necrotic material has been completely replaced by a granular or loose, reticulated, fibrillar structure. In other nodules a process of liquefaction necrosis appears to be in progress, and large, round or oval masses of necrobiotic tissue stand out from the background. These masses appear to represent remains of dense, fused, collagen bundles. At the center of many of the larger nodules a process of cystic degeneration is apparent and amorphous clumps of necrotic and necrobiotic tissue lie free in the center of large, cystic cavities (Fig. 12).

In addition to the disintegrating clumps of collagenous material the remains of other tissue elements can be observed in some of the necrotic areas. Occasionally scattered throughout these areas, but more frequently toward the periphery, are the chromatic remains of disintegrating cells. In some instances these are definitely the remains of polymorphonuclear leucocytes but others appear to be disintegrating, large, mononuclear cells. Occasionally, also, larger and smaller, branching, elastic fibers can be seen.

either killed nearly all animals within 48 hours after injection, or affected practically none. And in several experiments planned to compare the reaction of susceptible and resistant mice to these organisms so injected, the result was that either nearly all animals died, irrespective of breed, or all survived. These results may be interpreted as showing that the differences in inherent resistance of the tested mice are limited to the respiratory tract mucous membrane at the portal of entry and are not present in the interior tissues. Another



TEXT-FIG. 6. Relative susceptibility of four lines of mice to various infectious agents.

interpretation, more probable in our opinion, is that the organisms were too virulent and the method of testing too crude to permit a demonstration of any possible tissue differences which may be present similar to those in *B. enteritidis* infection.

DISCUSSION

The experiments in this and the preceding paper (1) are a step in the analysis of inherent resistance to infection. Heredity has proved clearly to be an element of fundamental importance in determining the

3. *Peripheral Area*.—Surrounding the cellular zone is a wide area of rather dense connective tissue. The connective tissue tends to be arranged circumferentially and consists of thick bundles of collagen fibers and more or less mature fibrocytes. Occasional, small, round cells are scattered throughout. It is this zone which apparently is largely responsible for the firm nodular character of the lesion in the gross and accounts for the relative ease with which the nodules may be removed. External to the zone of dense and mature scar tissue the lesion presents the more or less normal appearance of loose subcutaneous tissue.

Vascular Lesions of Subcutaneous Nodules in Rheumatoid Arthritis

In the peripheral areas of the nodules and in the surrounding connective tissue the blood vessels frequently show significant changes. These changes affect both the arterioles and capillaries although all the vessels in any one section are not uniformly involved.

The appearance of the affected vessels is again largely determined by the stage of the inflammatory process. The early and acute lesions present a picture which is strikingly similar to, if not identical with, that described by VonGlahn and Pappenheimer (14) as characteristic of the vascular lesions in rheumatic fever. A typical early vascular lesion from rheumatoid arthritis is shown in Fig. 17. In Fig. 17 it appears that the wall of the vessel is thickened in comparison with the caliber of the lumen. In this particular section the endothelium appears intact, but the intima is involved in the same type of peculiar fibrinoid swelling which constitutes such a prominent feature in the nodules already described. This fibrinoid material takes the usual fibrin stains, but its character and distribution, especially as seen in Masson preparations, tend to cast doubt on its true fibrin nature. A considerable portion of the material appears to consist of partially degenerated collagen. External to the area of fibrinoid swelling is a cellular tissue having a distinctive and peculiar character. Its appearance is well described in the article by VonGlahn and Pappenheimer on vascular lesions in rheumatic fever:

“External to the necrotic wall of the vessel is a cellular tissue having a very distinctive and peculiar appearance. It is composed of a loose fibrillar stroma, in part fibrinous, in which are many nuclei. One may distinguish (1) lobed nuclei of polymorphonuclear neutrophils, many of them pyknotic and fragmented, especially those nearest the vessel wall; (2) larger vesicular nuclei, staining less intensely than those of the polymorphonuclears and often distorted or compressed into bizarre elongate or club-shaped forms. They tend to be arranged radially. Still further out is a loose infiltration of lymphoid and plasma cells, occasional eosinophiles and young connective tissue cells.”

In other vessels the intima is greatly thickened by a proliferation of subendothelial cells. This process may involve the vessel wall in the whole of its circumference or only a portion of the wall may be affected leading to the formation of small projections into the lumen (Fig. 18). That this process, however, is not con-

4. Of four lines of mice relatively susceptible to *B. enteritidis*, three were likewise susceptible to *Pasteurella avicida*, *B. friedlaenderi*, and pneumococcus given intranasally. A fourth line was significantly more resistant. Lines of mice relatively resistant to *B. enteritidis* were likewise resistant to the three respiratory tract pathogens.
5. When *Pasteurella avicida*, *B. friedlaenderi*, and pneumococcus were injected intraperitoneally or intravenously, no significant differences in duration of life of the several lines of mice could be demonstrated.
6. Of four lines relatively susceptible to *B. enteritidis*, two were susceptible to an intranasal instillation of louping ill virus. Lines resistant to *B. enteritidis* proved relatively susceptible to the virus infection.

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nodules would be mistaken for rheumatic lesions. In the case of rheumatoid arthritis, the gross appearance of the nodular lesions more closely resembles those observed in syphilis and yaws and it becomes necessary to resort to histological studies to demonstrate significant differences. Through the courtesy of Dr. H. Hanford Hopkins and Dr. L. A. Brunsting, we have had the opportunity to study sections of several of the so called juxta-articular nodules in syphilis. Although the material at our disposal has been somewhat limited, it can be stated that the histological appearance of the nodular lesions in syphilis is not at all that of the characteristic lesions in rheumatoid arthritis. Crouzon and Bertrand (18) have recently reported similar conclusions. It is true that one of Hopkins' preparations presented the characteristic picture of a rheumatoid arthritis nodule, but this patient, in addition to suffering from syphilis, was also a victim of rheumatoid arthritis. In another of Hopkins' sections, from a patient with syphilis, there was present one area of focal necrosis surrounded by a zone of inflammatory cell infiltration. This area showed some resemblance to the lesions seen in rheumatoid arthritis, but the character of the cellular reaction was quite different. In particular the characteristic palisade formation of large mononuclear cells was conspicuously absent. Further distinctions between such gummatous lesions and the lesions observed in rheumatoid arthritis have recently been detailed by Schosnig (19). The appearance of the nodules in yaws, as described in the Harvard African Expedition (20), is so distinct as to leave no room for confusion. The opportunity has not presented itself for the examination of the lesions in acrodermatitis chronica atrophicans, but the recorded photographs of such lesions show few of the characteristic features of the lesions in rheumatoid arthritis. The nodular lesions so frequently seen in gout present such distinct characteristics that it is unnecessary to enter into any discussion of their appearance.

In our experience subcutaneous nodules of the type described have only been observed in cases of rheumatoid arthritis. Careful search for similar lesions has been made in several hundred cases of osteo-(hypertrophic, degenerative) arthritis and none has been found. It is believed that this observation lends support to the contention that this form of chronic multiple arthritis represents a separate and distinct

A COMPARATIVE STUDY OF SUBCUTANEOUS NODULES IN RHEUMATIC FEVER AND RHEUMATOID ARTHRITIS*†

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PLATES 50 TO 55

(Received for publication, February 1, 1933)

Since the original observations of Meynet (1) and Barlow and Warner (2) on the occurrence of subcutaneous nodules in rheumatic fever, numerous studies on these lesions have appeared. It is now well recognized that subcutaneous nodules are a frequent manifestation of the disease rheumatic fever and that these lesions present a highly characteristic histological structure. Occasional reference (3) has also been made to the presence of subcutaneous nodules in rheumatoid arthritis but, until recent years, detailed pathological descriptions of the lesions in this disease have not been presented. In the last few years, however, communications have appeared (4-7), which have shown that subcutaneous nodules are of not infrequent occurrence in rheumatoid arthritis and that these lesions also possess a highly characteristic histological structure. These observations have led to a comparative study of the nodules in the two diseases and to a consideration of the relationship between the clinical entities, rheumatic fever and rheumatoid arthritis (3).

In the present communication there is presented a detailed study of the subcutaneous nodules in the two diseases. This study has

* This paper was presented in abstract before the twenty-third annual meeting of the American Society for Clinical Investigation, Atlantic City, May, 1931.

† The term rheumatoid arthritis is used synonymously with the terms chronic infectious and atrophic arthritis.

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The author wishes to express his thanks to Professor A. M. Pappenheimer of the Department of Pathology of the College of Physicians and Surgeons, Columbia University, for his kind assistance in this study.

Addendum.—While the foregoing investigation was being carried out, there appeared an extensive pathological study of tissue changes in rheumatic fever and rheumatoid arthritis by Klinge and Grzimek (*Virchows Arch. path. Anat.*, 1932, 284, 646). These authors state:

"So we are able to explain without difficulty on the basis of our material the close relationship of chronic polyarthritis, infectious arthritis, polyarthritis lenta with acute febrile rheumatism (acute joint rheumatism) as a continuous sequence of one disease process with different phases and with different clinical and anatomical manifestations in each individual phase."

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Rheumatic Fever

attain considerably larger dimensions. In our experience the size of the nodules bears some relation to the period of their duration and to the age of the patient.

Duration.—As a rule the nodules persist for a matter of weeks only, but several cases have been observed in which they remained for many months.

Type of Case and Prognosis.—The appearance of nodules is generally associated with a severe form of the disease and usually is indicative of cardiac damage.

Other Characteristics.—The nodules are located in the subcutaneous tissue and show no evidence of attachment to the overlying skin. Not infrequently they appear to be attached to the sheaths of the underlying tendons and occasionally to the periosteum of the underlying bone. The skin is freely movable over them and palpation of the lesion frequently conveys the sense of a hard foreign body. They are not associated with pain except when their situation exposes them to undue pressure.

From the foregoing description it is apparent that the gross appearance and clinical characteristics of the nodules in the two conditions, rheumatic fever and rheumatoid arthritis, are strikingly similar. In the following section, a comparison of the microscopic appearance of the lesions in the two diseases is presented.

Rheumatoid Arthritis

rheumatic fever. They usually vary in size between 1 and 2 cm. in diameter, but smaller ones are frequently found. This appears to be particularly true in the case of younger individuals.

Duration.—In the majority of cases the nodules persist for a matter of months or years. It is not unusual, however, to observe the smaller ones disappear in the course of a few weeks.

Type of Case and Prognosis.—In our experience subcutaneous nodules of the type described have only been observed in that form of arthritis to which the terms rheumatoid, chronic infectious and atrophic are variously applied. In spite of diligent search, we have not found similar lesions in cases of osteo-(hypertrophic, degenerative) arthritis. The presence of nodules in rheumatoid arthritis almost without exception indicates a severe form of the disease and, as a rule, their persistence is associated with a bad prognosis.

Other Characteristics.—The other general characteristics of the nodules in rheumatoid arthritis are of the same nature as those in rheumatic fever. The lesions are located in the subcutaneous tissue and are not attached to the overlying skin. They not infrequently appear to be attached to tendon sheaths and occasionally bear a definite association with the walls of bursae. The nodules are not painful and only in occasional instances do they cause the patient any inconvenience.

PLATE 53

FIG. 9. Rheumatic fever nodule. Area of focal necrosis which involves collagen bundles in a peculiar hyalin degeneration. Hematoxylin and eosin stain. $\times 280$.

FIG. 10. Rheumatoid arthritis nodule. Area of focal necrosis showing a similar type of hyalin degeneration of collagen. Note the characteristic radial distribution of the large mononuclear cells. Hematoxylin and eosin stain. $\times 280$.

FIG. 11. Rheumatic fever nodule. Papillary villus-like structure projecting into area of cystic degeneration. Cell outlines frequently indefinite and fusion of cells into multinucleated elements. Occasional mitotic figures. Masson's trichrome stain. $\times 80$.

FIG. 12. Rheumatoid arthritis nodule. Note that description of Fig. 11 applies equally well to this section. $\times 80$.

PLATE 54

FIG. 13. Rheumatic fever nodule. Large mononuclear cells at margin of area of necrosis. Hematoxylin and eosin stain. $\times 630$.

FIG. 14. Rheumatoid arthritis nodule. Large mononuclear cells at margin of area of necrosis. Hematoxylin and eosin stain. $\times 630$.

FIG. 15. Rheumatic fever nodule. Multinucleated giant cells at margin of area of necrosis. These cells appear to surround fragments of degenerated collagen. Masson's trichrome stain. $\times 400$.

FIG. 16. Rheumatoid arthritis nodule. Multinucleated giant cells at margin of area of necrosis. Compare with Fig. 15. Masson's trichrome stain. $\times 400$.

PLATE 55

Vascular lesions of subcutaneous nodules in rheumatoid arthritis.

FIG. 17. Acute vascular lesion. Note necrosis of vessel wall and type of inflammatory tissue surrounding the vessel. Hematoxylin and eosin. $\times 280$.

FIG. 18. Intimal hyperplasia; splitting of elastica. Elastic tissue stain. $\times 280$.

FIG. 19. Intimal hyperplasia; formation of a new internal elastic membrane. Elastic tissue stain. $\times 280$.

FIG. 20. Arteritis verrucosa; polypoid projection into lumen of vessel. Elastic tissue stain. $\times 280$.

be made of the fact, however, that mitotic figures not infrequently appear in considerable numbers. The tendency of these cells to fuse together and form multinucleated giant cells is well known.

The course and evolution of the nodules is further understood by a consideration of the larger and probably older lesions. Microphotographs of such nodules appear in Figs. 9 and 11. In Fig. 9 is shown a rheumatic nodule with a larger area of central necrosis but otherwise presenting all the characteristic features of the early lesions. In occasional instances the center of a large nodule undergoes cystic degeneration. In Fig. 11 is shown a section at the margin of the area of cystic degeneration with hyperplastic villus-like structures projecting into the lumen. In Fig. 15 the appearance of numerous multinucleated giant cells is well shown. These cells present the characteristics of the foreign body type and one gathers the impression that their appearance is associated with the removal of necrotic, tissue fragments.

The terminal stages in the evolution of the rheumatic nodule appear to consist in the formation of scar tissue without distinctive features.

This brief review of the histogenesis of the rheumatic nodule serves to show that various stages in the development of the lesions are characterized by certain morphological distinctions, but that the essential features are the same throughout.

Microscopic Appearance of Subcutaneous Nodules in Rheumatoid Arthritis

The study of the subcutaneous nodules in rheumatoid arthritis has revealed that the histological structure of these lesions presents a uniform and highly characteristic appearance which is closely related to, if not identical with, that observed in rheumatic fever.

In Fig. 6 is presented a low power magnification of a typical nodule from a case of rheumatoid arthritis in a man of 50 years. This nodule was situated over the olecranon process and had been present for 2 years at the time of excision. It is seen that, as in rheumatic fever, the lesion consists of multiple conglomerate areas of varying sizes and shapes. Each individual area is composed of three more or less well defined zones which closely resemble those described in the rheumatic fever lesions. These zones consist of (1) a large and irregular area of central degeneration, (2) a surrounding richly cellular tissue, the cells of which are disposed in characteristic radial fashion, and (3) an enclosing area of rather dense and avascular fibrous connective tissue.

The character of the area of central necrosis presents great variation in different nodules. Its appearance is in part determined by the size of the area involved and in part by the duration of the process. In the smaller and apparently earlier

In many sections the margin of the necrotic area is well defined but in other sections it gradually merges with the surrounding tissue and in many such instances this tissue appears to be progressively involved in the necrobiotic process.

In the case of some of the larger nodules the necrotic material has undergone further alterations. It has lost its homogeneous appearance and is characterized by the deposition of numerous, large, rhomboidal crystals. These crystals are apparently composed of cholesterol. In none of the lesions which we have had the opportunity of studying has calcium deposition or osteoid tissue formation been observed, but such an appearance has been described by others.

Further details of the nature of the area of central necrosis are brought out by the aid of Laidlaw's silver impregnation stain. These preparations reveal that the reticular framework underlying the area of fibrinoid swelling is relatively well preserved and participates in the radial character of the lesion. With this stain identical pictures are observed in the nodules from rheumatic fever and rheumatoid arthritis. Typical sections are presented in Figs. 3 and 4.

2. *The Cellular Zone*.—Surrounding the central necrotic area is a zone of cellular tissue, the elements of which tend to be characteristically arranged in radial fashion (Figs. 10 and 12). In their general appearance the cells of this tissue suggest epithelioid cells at the margin of tubercles. The cells have a fusiform or polygonal shape and frequently the cytoplasm is prolonged into multinucleate elements. Often the cell outlines are indefinite and fusion into several slender processes. The nuclei are large, oval or lobate with a distinct nuclear membrane and one or several conspicuous nucleoli; with two or several nuclei is frequently observed. Although the cell outlines are frequently indistinct, each element is surrounded by a wavy, pink-staining, reticular fibril. Characteristically disposed in radial fashion, these epithelioid cells are often heaped up in a curious, palisade fashion several cells deep. In some places at the margin of the necrotic area the cells surround clumps of disintegrating material, forming large foreign body giant cells (Fig. 16). One gathers the impression that this change occurs only in those nodules in which the integrating process has ceased and the foreign material is being resorbed.

The intercellular substance consists of fibrillar stroma composed of delicate reticular fibers. The coarse fibers stain red in Van Gieson preparations; the more tenuous ones are well seen in aniline blue preparations.

Varying greatly in different sections there occurs throughout this area an inflammatory cell infiltration chiefly consisting of lymphocytes and monocytes. In some sections considerable numbers of polymorphonuclear leucocytes are also seen and among these scattered elements of the cellular zone appear to be in active stages of proliferation and mitotic figures are frequently seen. In some of those nodules in which the central area has undergone cystic degeneration the marginal cellular wall displays evidence of active hyperplasia and in many places projects in papillary fashion into the cyst-like cavities (Fig. 12).

lined to the intima is frequently shown by the presence of a well developed layer of elastic fibers extending into such verrucous projections (Fig. 20). In other sections splitting of the elastica has occurred and, in a few instances, an entirely new elastic membrane has been formed. In some vessels clefts or spaces, lined with endothelium and containing red blood cells, appear in the hyperplastic intima. This secondary vascularization of the intima presents a picture very similar to that described by VonGlahn and Pappenheimer in the vascular lesions of rheumatic fever.

The adventitia of many vessels shows a well marked perivascular cell infiltration by monocyctic cells, small round cells and scattered plasma and mast cells. In addition to this infiltration by wandering cells there is frequently a definite hyperplasia of histiocytic cells which apparently take their origin from the adventitia. These cells appear in all respects similar to the epithelioid cells which occur at the margin of the necrotic areas of the nodules. For the most part they are disposed in concentric fashion and frequently envelop the vessel in a mantle several cells deep. In some instances, zones of capillary congestion occur about the affected vessels.

Occasional vessels are seen with old and thickened walls in which the various coats cannot be readily distinguished. Such vessels probably represent the terminal stages of the vascular lesions.

Bacteriological Investigations on Subcutaneous Nodules in Rheumatoid Arthritis

The results of bacteriological studies on subcutaneous nodules in rheumatoid arthritis have been reported elsewhere (15). Attempts to culture bacteria from these lesions have been uniformly unsuccessful. In addition, efforts to demonstrate the presence of bacteria in sections of the nodules have met with consistent failure.

DISCUSSION

In order that conclusions can be drawn as to the significance of characteristic lesions in any particular disease, it is first necessary to show that such lesions occur only in the particular disease in question. In the case of the subcutaneous nodules in rheumatoid arthritis and rheumatic fever, it cannot be claimed that such lesions, as judged by their gross appearance alone, are in any way specific for these diseases. It is well known that subcutaneous nodular lesions are not infrequently observed in other conditions such as syphilis, yaws, gout, tuberculosis and acrodermatitis chronica atrophicans (16, 17). However, as Ewen (11) has recently pointed out, it is most improbable that such

disease entity (21). In a search for similar manifestations in other conditions nodular lesions have been examined whenever they could be found in patients suffering from a variety of other diseases. Four such nodules were excised from patients suffering from various conditions. One of these proved to be a so called giant cell sarcoma of a tendon sheath, another a xanthoma, a third a lipoma and the fourth a fibromyxoma. On the other hand, it should be pointed out that Findlay (8) has recently reported the presence of a characteristic rheumatic nodule in a child in whom no other stigmata of the rheumatic infection could be observed. Furthermore, in certain other acute infections, Holsti (22) has described vascular lesions which resemble those seen in rheumatic fever. Further investigation is therefore necessary before the lesions which have been described can be considered as absolutely specific for rheumatic fever and rheumatoid arthritis. In the present state of knowledge it would be more correct to regard both the nodular and vascular lesions as highly characteristic of these respective diseases.

SUMMARY

The foregoing comparative study on the subcutaneous nodules in rheumatic fever and rheumatoid arthritis is presented as part of an investigation which has been conducted in this clinic on the relationship of the two clinical entities, rheumatic fever and rheumatoid arthritis. It is believed that the present study has shown that these lesions are highly characteristic of the two diseases and that they represent different phases of the same, fundamental, pathological process. However, it should be pointed out that the presence of closely related or even identical lesions in two, separate, clinical entities cannot be considered as valid evidence in support of the hypothesis that the two diseases are etiologically related. Comparative clinical studies on the relationship of rheumatic fever and rheumatoid arthritis will be presented in a succeeding communication. These studies, as well as serological investigations on the two diseases which have been reported elsewhere (23, 24), lend further support to the conception that rheumatic fever and rheumatoid arthritis are intimately related and possibly different responses of affected individuals to the same etiological agent.

M. H. DAWSON

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EXPLANATION OF PLATES

PLATE 50

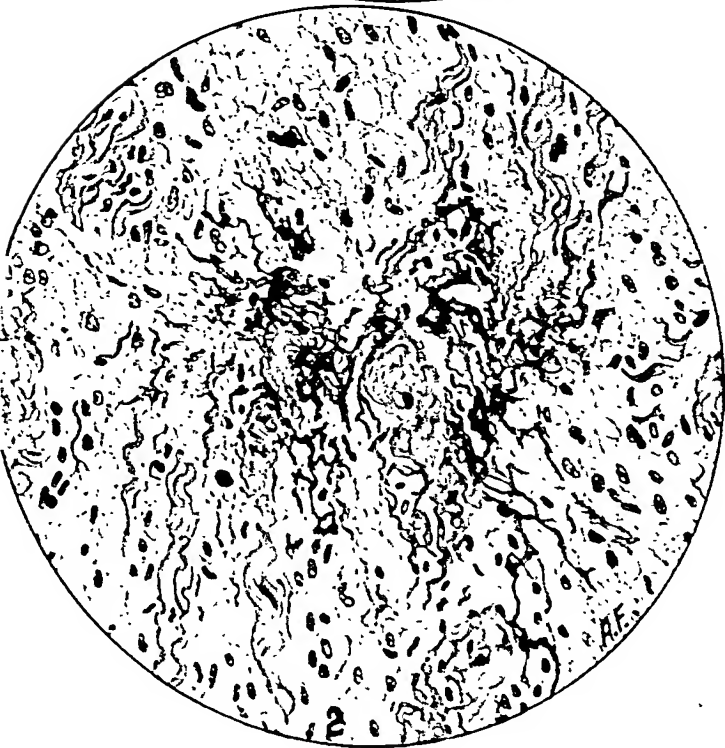
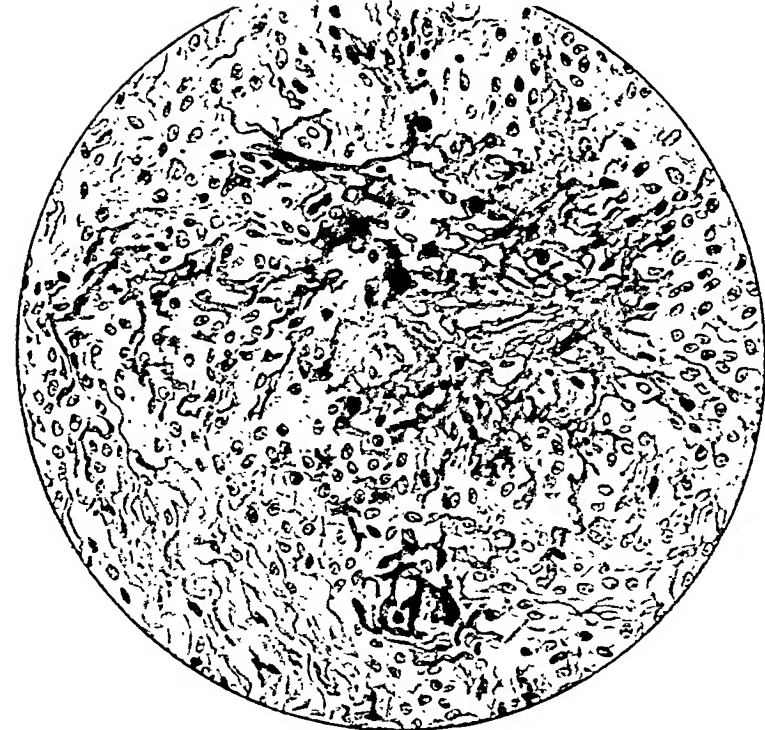
- FIG. 1. Rheumatic fever nodule. Area of focal necrosis surrounded by large mononuclear cells which tend to be arranged in radial fashion. The necrotic material appears to be composed largely of degenerated bundles of collagen which stain a brilliant red. The normal collagen at the periphery accepts a vivid green stain. Note mitotic figure near left border. Masson's trichrome stain. $\times 280$.
- FIG. 2. Rheumatoid arthritis nodule. Area of focal necrosis surrounded by large mononuclear cells. This is a very early lesion and is shown for comparison with the rheumatic fever nodule, the description of which applies equally well to this section. The appearance of larger and older lesions is shown in subsequent illustrations. Masson's trichrome stain. $\times 280$.

PLATE 51

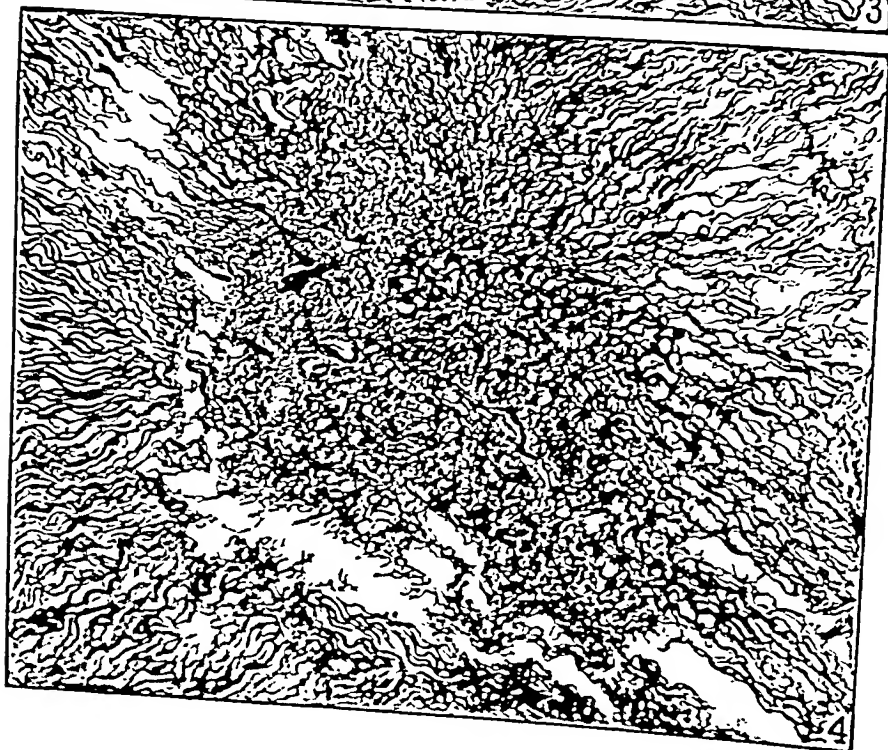
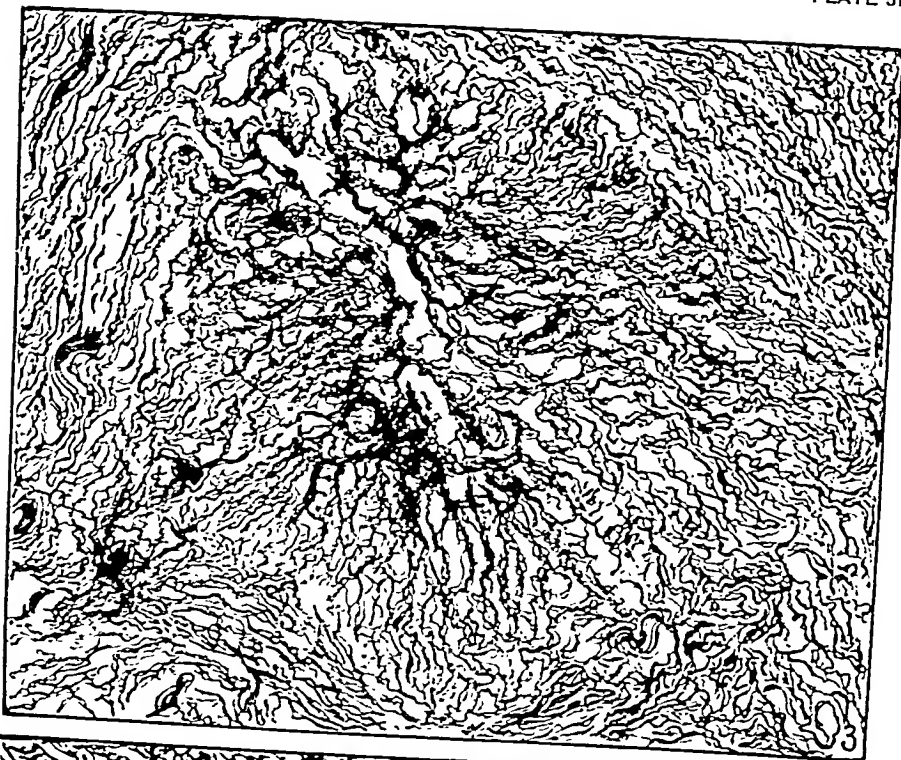
- FIG. 3. Rheumatic fever nodule. Radiating reticular structure throughout area of focal necrosis. Laidlaw's silver impregnation stain. $\times 80$.
- FIG. 4. Rheumatoid arthritis nodule. Radiating reticular structure throughout area of focal necrosis. Reticulum is more dense than in rheumatic fever nodule but otherwise similar. Laidlaw's silver impregnation stain. $\times 80$.

PLATE 52

- FIG. 5. Rheumatic fever nodule. Multiple areas of focal necrosis surrounded by cellular zones and enclosed by fibrous connective tissue. Masson's trichrome stain. $\times 30$.
- FIG. 6. Rheumatoid arthritis nodule. Larger and more definite areas of focal necrosis surrounded by dense fibrous connective tissue. Masson's trichrome stain. $\times 18$.
- FIG. 7. Rheumatic fever nodule. A small area of focal necrosis surrounded by large mononuclear cells. The necrotic material is composed of degenerating bundles of collagen. Masson's trichrome stain. $\times 280$.
- FIG. 8. Rheumatoid arthritis nodule. A small area of focal necrosis surrounded by large mononuclear cells. Note similarity to Fig. 7. Masson's trichrome stain. $\times 280$.



(Dawson: Rheumatic fever and rheumatoid arthritis)



(Dawson: Rheumatic fever and rheumatoid arthritis)

TABLE I

Rabbits Sensitized with Horse Serum. Hemorrhagic Necrosis in Skin Sites Prepared with Bacterial Filtrate and Reinjected with Horse Serum

No. of rabbits	Intravenous sensitizing injections	Skin-preparatory injections			Time between sensitizing and preparatory injections <i>days</i>	Skin test injections			Time between preparatory and test injections <i>hrs.</i>	Results*		
		Upper right quadrant	Lower right quadrant	Upper left quadrant		Upper right quadrant	Lower right quadrant	Upper left quadrant		Upper right quadrant	Lower right quadrant	Upper left quadrant
2	1 cc. horse serum	0.25 cc. <i>B. typhosus</i> Filtrate 1776	0.5 cc. histamine diluted 1:100	—	7	0.5 cc. horse serum	0.5 cc. horse serum	—	24	2/0	0/2	—
2	"	"	0.5 cc. horse serum	—	7	"	"	—	24	2/0	0/2	—
3	"	"	"	—	7	"	"	—	6	0/3	0/3	—
3	"	0.5 cc. horse serum	0.25 cc. <i>B. typhosus</i> Filtrate 1776	—	7	<i>B. typhosus</i> Filtrate 1776	"	—	24	0/3	3/0	—
3	"	0.25 cc. <i>B. typhosus</i> Filtrate 1776	—	—	4	0.5 cc. horse serum	—	—	24	0/3	—	—
3	"	"	0.25 cc. <i>B. typhosus</i> Filtrate 1776	0.25 cc. <i>B. typhosus</i> Filtrate 1776	7	"	0.5 cc. human serum	0.5 cc. sheep serum	2	3/0	± 1/2	0/3
3	"	"	"	"	7	"	0.5 cc. guinea pig serum	"	2	3/0	0/3	0/3

± = doubtful reaction.

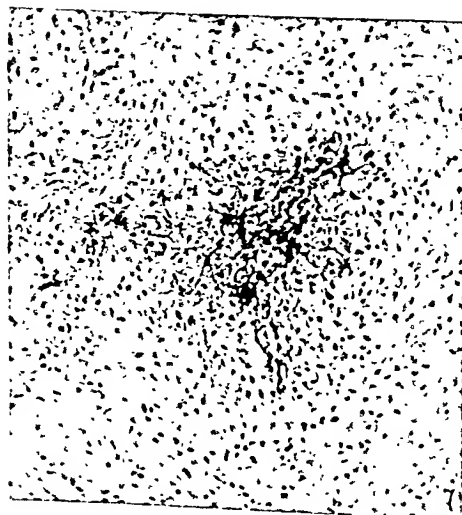
* The numerator indicates the number of skin sites showing severe hemorrhagic necrosis; the denominator the number of negative areas.



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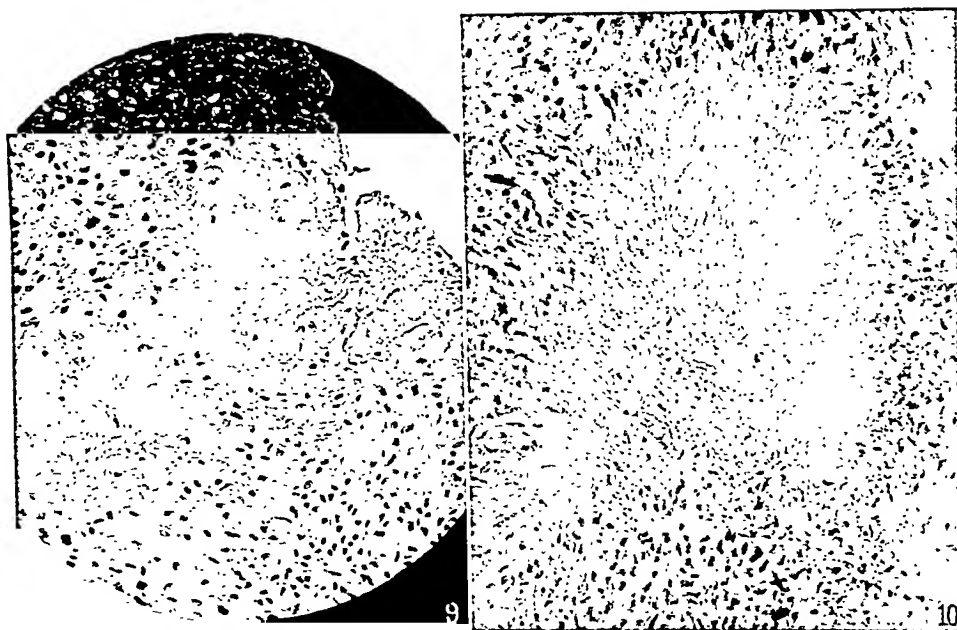


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(Dawson: Rheumatic fever and rheumatoid arthritis)

TABLE III
Hemorrhagic Necrosis in Skin Sites Prepared with Bacterial Filtrates in Passively Sensitized Rabbits

No. of rabbits used	First intradermal injection		Second intradermal injection		Time between intradermal injections	Dose and material of intravenous injections per kilo of body weight	Time of intravenous injections	Results*	
	Upper right quadrant	Lower right quadrant	Upper right quadrant	Lower right quadrant				Upper right quadrant	Lower right quadrant
6	0.25 cc. meningococcus Group III Filtrate 1797	—	0.5 cc. Antipneumococcus Type III horse serum	—	24 hrs.	2 cc. Pneumococcus Type III filtrate	Simultaneously with 2nd intradermal injection	6/0	—
3	"	—	0.5 cc. horse serum	—	24	"	"	0/3	—
3	"	—	0.5 cc. Antipneumococcus Type I horse serum	—	24	"	"	0/3	—
3	"	—	0.5 cc. Antipneumococcus Type II horse serum	—	24	"	"	0/3	—
3	"	—	0.5 cc. Pneumococcus Type I filtrate	—	24	2 cc. Antipneumococcus Type III horse serum	"	1/2	—
3	"	—	0.5 cc. Antipneumococcus Type III horse serum	—	6	2 cc. Pneumococcus Type III filtrate	24 hrs. after 1st intradermal injection	0/3	—
3	0.5 cc. Antipneumococcus Type III horse serum	—	0.25 cc. Meningococcus Group III Filtrate 1797	—	24	—	—	0/3	—



(Dawson: Rheumatic fever and rheumatoid arthritis)

paratory effect of a potent bacterial filtrate. The results were negative if the injections immediately followed each other or were carried out within 6 hours. The interval of time successfully employed was 24 hours. Intervals of time between 6 and 24 hours have not been studied as yet.

The sensitization of rabbits was accomplished by single intravenous injections 7 days prior to the tests. Shorter incubation periods were insufficient (*i.e.* 3 days).

The reaction observed was specific. Reinjection of prepared areas with sheep, guinea pig and human serum in rabbits sensitized by single injections of horse serum elicited no reactions.

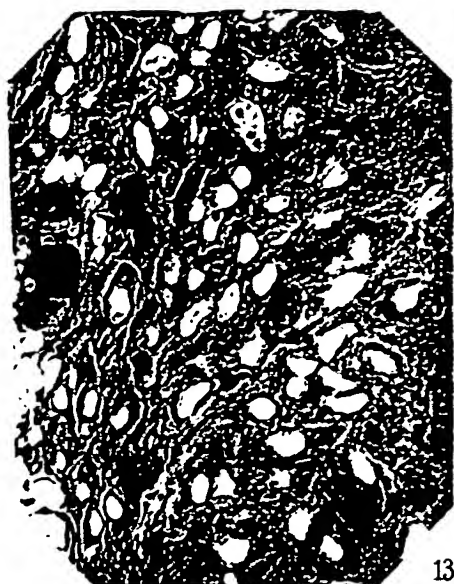
Some of the rabbits were bled on the day of preparatory injections and the sera tested for precipitins against horse serum. The results are recorded in Table II.

As is seen from Table II, there was no apparent parallelism between the precipitin titers of the various sera and the incidence of the reactions. These were easily obtained in most of the rabbits tested in the manner described. Occasionally rabbits failed to give the phenomenon. The percentage of negative animals has not been ascertained as yet.

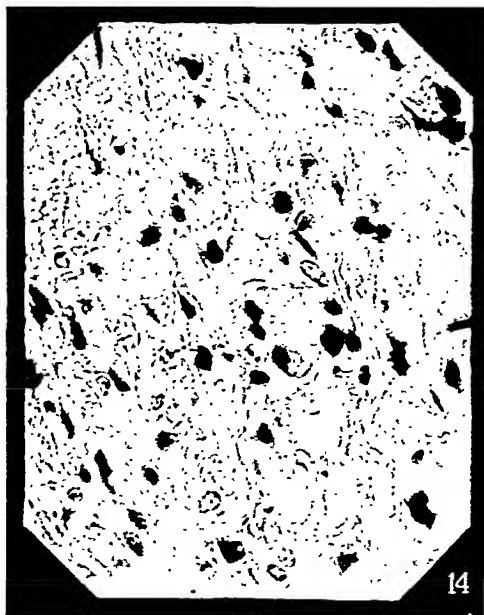
The experiments recorded in Table III were carried out in order to determine whether the reaction could be passively transferred to normal rabbits.

The antibodies employed were anti-horse rabbit sera, anti-human horse sera and Antipneumococcus Type III horse sera. The antigens were normal rabbit and human sera and Pneumococcus Type III culture filtrate. The anti-horse and anti-human sera were made according to methods described previously (1, 3). The antipneumococcus sera were obtained from the New York Board of Health through the courtesy of Miss G. Cooper. The pneumococcus antigen was a filtrate of a culture in meat infusion broth pH 7.8 containing 0.3 per cent glucose and 0.3 per cent maltose. On repeated retests, this filtrate proved incapable of eliciting any reaction when injected intravenously, in a dose of 4 cc. per kilo of body weight, into rabbits prepared with the *B. typhosus* and meningococcus filtrates. In the experiments described here, a dose of only 2 cc. was employed.

One or two skin sites were each injected with 0.25 cc. of *B. typhosus* or meningococcus "agar washings" filtrate. 24 hours later the prepared skin sites were injected with the antibody-containing sera and simultaneously injected intravenously with the homologous antigens. 4 to 5 hours after the intravenous injection there appeared severe hemorrhagic necrosis in the injected skin sites.



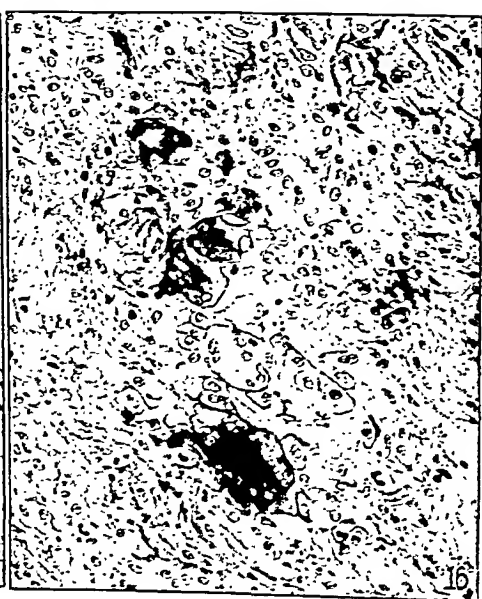
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(Dawson: Rheumatic fever and rheumatoid arthritis)

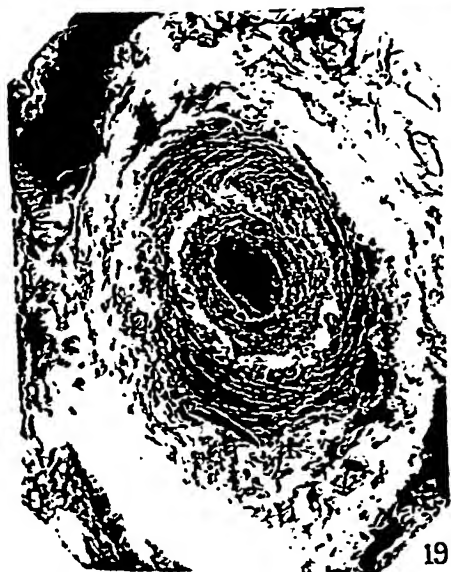
human horse serum employed in these experiments, whilst the latter diluted 1:40 failed to precipitate with undiluted 1:10 human serum. As shown by Opie (4), large amounts of antibody are required for passive transfer of the Arthus phenomenon. Inasmuch as, in addition, the antibody disappears rapidly from the circulation, it is easily understood why it was necessary to introduce a large amount of antibody in the experiments described above.

In passively sensitized rabbits no incubation period was required after the injection of the antibody. The experiments were consistently successful if the antigen and the antibody were injected simultaneously in the manner described. If some time was allowed to elapse between the injections, the outcome of the experiments was negative.

The consensus of opinion is that passive transfer of bacterial hypersensitiveness has not been definitely demonstrated as yet (Doerr, Zinsser (7)). Some claims are suggestive (Bail, Helmholz, Zinsser and Mueller, Gay and Claypole, Meyer and Christiansen, Julianelle (8)) but, as stated by Zinsser (7), they do not possess the convincing regularity of passive transfer of anaphylaxis. The experiments embodied in this paper demonstrate that passive transfer of hypersensitiveness to a bacterial antigen (*i.e.* pneumococcus) can be easily accomplished provided the interaction of passively acquired antibodies with the antigen takes place intravascularly at the site of a tissue of induced vulnerability.

The specificity of the antigen-antibody interaction has been demonstrated. There was observed serological group specificity in experiments with pneumococcus filtrate.

The state of vulnerability was induced by certain bacterial filtrates, the skin-preparatory potency of which was proved by titrations (5). It could not be induced with horse serum, meat infusion broth and streptococcus culture filtrates in rabbits sensitive to horse serum. In order to obtain the vulnerable state, in addition to employing potent bacterial filtrates, it was necessary to allow a definite period to elapse, the optimum period being 24 hours, 6 hours being insufficient. Thus, there was clearly demonstrated that the *sine qua non* of the phenomenon is that a state of vulnerability be induced at the site of the antigen-antibody interaction. This change elicited by bacterial filtrates



(Dawson: Rheumatic fever and rheumatoid arthritis)

In the attempt to utilize the above facts for the explanation of focal and skin reactions of bacterial hypersensitiveness, it may be assumed that the infected foci being in a state of vulnerability throw off soluble bacterial products which induce the state also in the skin of the infected animal. Upon parenteral introduction of the specific antigen there would occur an intravascular interaction between the injected antigen and the antibodies acquired in the course of the infection, with the resulting formation of toxic principles. The toxic principles would elicit injury in infected tissues (*i.e.* focal reactions). In intradermal tests, the toxic principles produced through the interaction of the locally injected antigen and circulating antibodies would elicit a local reaction (*i.e.* bacterial skin hypersensitiveness). It might be expected that the severity of focal and local reactions would depend on the degree of vulnerability of the infected tissues and of the skin of the infected animal and also on the amount of toxic principles formed, which in turn would depend on the titer of actively acquired antibodies and amount of antigen injected.

It seems that the introduction of the notion of cell vulnerability as essential prerequisite for response to specific antigen-antibody interaction together with the demonstration of intravascular toxic principles resulting from the interaction, offer new possibilities for studies on bacterial hypersensitiveness.

SUMMARY

The observations reported in this paper demonstrate that the intravascular interaction of bacterial and animal protein antigens with homologous antibodies at the site of a tissue made vulnerable by bacterial filtrates induces prompt severe hemorrhagic necrosis in this tissue. In the light of these observations there is offered an explanation of the mechanism underlying focal and skin bacterial hypersensitiveness.

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PHENOMENON OF LOCAL SKIN REACTIVITY TO BACTERIAL FILTRATES IN ITS RELATION TO BACTERIAL HYPERSENSITIVENESS

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PLATE 56

(Received for publication, January 16, 1933)

Observations reported in previous communications (1-3) allowed differentiation of two distinct and separate phases of the phenomenon of local skin reactivity to bacterial filtrates: (a) the change in tissue elicited by the preparatory injection of potent bacterial filtrates whereby it becomes susceptible to certain toxic principles, and (b) the injurious effect of the principles upon the vulnerable tissue. The toxic principles are bacterial substances identical or closely related to true exotoxins; mixtures of animal proteins with homologous antisera; and mixtures of bacterial antigens, incapable of eliciting the phenomenon by themselves, with homologous antisera. The intradermal reinjection of antigen-antibody mixtures into an area prepared with a bacterial filtrate produces no effect. In order to obtain a severe reaction at the prepared skin site, it is necessary to inject intravenously either the antigen-antibody mixture or to inject intravenously the antigen and the antibody separately. Intravenous injection of the antigen alone is sufficient to elicit reactions at the prepared sites of rabbits possessing actively acquired antibodies. In the experiments described below it was deemed of interest to determine whether the reaction could also take place if either the antigen or the antibody was present in the blood stream at the time when antibody or antigen was injected directly into the area previously prepared with the bacterial filtrate.

EXPERIMENTAL

As seen from the experiments summarized in Table I, rabbits were sensitized by single intravenous injections of normal horse serum, in a dose of 1 cc. per kilo

of body weight. 1 week later they received injections of *B. typhosus* "agar washings" filtrate into one to three areas of the skin of the abdomen (*i.e.*, upper and lower right and upper left quadrants, respectively). 24 hours afterwards the same skin areas were reinjected with normal horse serum. From 4 to 5 hours later the rabbits showed severe hemorrhagic necrosis at the site of the intradermal injections. The lesions were identical with those observed in the phenomenon of local skin reactivity to bacterial filtrates. Histological changes were also similar to those previously described (Fig. 1).

TABLE II

Precipitation Reactions with Sera of Rabbits Showing Hemorrhagic Necrosis upon Injection of Horse Serum into Vulnerable Skin Sites

Rabbit No.	Sensitizing injection per kilo of body weight	Time between sensitizing and test injections	Preparatory injections	Test injection	Time between preparatory and test injections	Intensity of hemorrhagic necrosis	Precipitation tests with various horse serum dilutions*				
							Undiluted	1:10	1:100	1:1000	1:10,000
9-57	Horse serum 1 cc.	7 days	Meningococcus Group III filtrate	Horse serum	24 hrs.	Strongly positive	±	4+	4+	4+	—
3-36	" "	7	" "	" "	24	" "	1+	1+	—	—	—
2-75	" "	7	" "	" "	24	" "	4+	4+	4+	1+	—
4-80	" "	7	" "	" "	24	No reaction	±	4+	4+	1+	—
1-07	" "	7	" "	" "	24	Strongly positive	2+	4+	4+	4+	1+

Amount of precipitate is recorded by plusses. ± indicates a slight turbidity. — shows absence of precipitation.

* The rabbit serum was used undiluted.

As Table I shows, injection of normal horse serum into skin areas prepared 24 hours previously by injection of horse serum, histamine diluted 1:1000 and sterile meat infusion broth did not elicit any hemorrhagic necrosis. Moreover, the horse serum failed to elicit the reaction in areas prepared with bacterial filtrates devoid of skin-preparatory potency (*i.e.* *Streptococcus viridans* culture filtrate) (Figs. 2 and 3).

It was necessary to allow a definite incubation period for the pre-

3	0.25 cc. meningococcus Group III Filtrate 1797	—	0.5 cc. rabbit serum	—	24	10 cc. horse serum	Simultaneously with 2nd intradermal injection	0/3
3	"	0.25 cc. <i>B. typhosus</i> Filtrate 1792	0.5 cc. horse serum	0.5 cc. horse serum	24	—	"	0/3 0/3
3	"	—	—	—	—	5.1 cc. of mixture of 1 part of horse serum and 49 parts of anti-horse rabbit Serum S42	24 hrs. after intradermal injection	3/0
3	"	—	0.5 cc. anti-horse rabbit Serum S42	—	24	10 cc. horse serum	Simultaneously with intradermal injection	3/0
3	"	—	"	—	24	20 cc. horse serum	"	3/0
3	0.5 cc. <i>B. typhosus</i> Filtrate 1792	—	0.5 cc. anti-human horse serum	—	24	2 cc. human serum	"	3/0
3	"	0.25 cc. <i>B. typhosus</i> Filtrate 1792	0.5 cc. human serum	0.5 cc. human serum diluted 1:10	24	6 cc. anti-human horse Serum 486	"	0/3 0/3
3	"	"	"	"	24	6 cc. anti-human horse Serum 486 diluted 1:10	"	0/3 0/3
3	"	—	"	—	24	20 cc. anti-human horse Serum 486	"	3/0

* The numerator indicates the number of skin sites showing severe hemorrhagic necrosis; the denominator the number of negative areas.

Another change in the technique of Noguchi consisted in the inoculation of four tubes of leptospira medium, one with a fragment of conjunctival tissue and the other three with a suspension of ground tissue, undiluted and in dilutions of 1:10 and 1:100 respectively. Separate plates were seeded with undiluted, 1:10 and 1:100 dilutions of the tissue suspension. It was found advantageous to inoculate, in addition, one or two tubes containing about 5 cc. of hormone broth with the undiluted suspension of the tissue. After growth was obtained, plates were seeded with the material for the purpose of identification of the organisms.

We should state here that for bacterial cultivation conjunctival secretions, or material of expressed follicles, are not as suitable as ground tarssectomized tissue.

Results of Cultivation Tests

The conjunctival tissue derived from folliculosis monkeys yielded in cultures a variety of microorganisms. Among those most frequently recovered were diphtheroids, staphylococci, chromogenic Gram-negative bacilli, organisms of the *subtilis* group, *Sarcinae*, and moulds. Of these microorganisms, 24 different strains were injected subconjunctivally in 47 monkeys without specific effect. The conjunctival tissues of 6 of the inoculated animals were again cultured and yielded no special organisms. *Bacterium granulosis*, however, was not recovered from cases of spontaneous folliculosis in 50 monkeys studied especially with the object of isolating this bacterium. In general, the bacteria were of the same species as were found both in monkeys having granular conjunctivitis following inoculation of trachomatous materials, and in normal animals, although in the latter instances the organisms were present less frequently and regularly.

In addition to these innocuous varieties of bacteria, we have isolated and cultivated from spontaneous conjunctival folliculosis of *Macacus rhesus* monkeys a hitherto undescribed microorganism that is capable of reproducing a folliculosis disease in normal *rhesus* monkeys and chimpanzees indistinguishable from the spontaneous affection. It has been recovered thus far from seven of ten animals having folliculosis, and has not been isolated from thirty-two monkeys which were either normal, inoculated with the aforementioned indifferent bacteria, or affected with experimental trachomatous conjunctivitis. The tissues derived from five cases of human trachoma also failed to yield this organism.

In other experiments the antigens were injected into the skin and the antibodies intravenously. These experiments were positive provided a sufficiently large amount of antibody was injected intravenously. Thus, a dose of 6 cc. per kilo of body weight, of anti-human horse serum gave negative results, whilst 10 cc. gave strong reactions. Also a dose of 10 cc. per kilo of body weight of anti-horse rabbit serum was necessary for the elicitation of the reaction.

A number of experiments, some of which are recorded in Table III, clearly demonstrated the specificity of the passive transfer described. In the work with *Pneumococcus* Type III filtrate, there was observed serological type specificity as well.

No incubation period was required for the passive sensitization, inasmuch as the experiments were successful when the injections of the antibody and the antigen were made simultaneously. As in the case of actively sensitized rabbits (page 861) it was necessary to allow a definite incubation period for the preparatory effect of the bacterial filtrates.

RESUMÉ AND DISCUSSION

The observations reported in this paper demonstrated that antigen-antibody interaction in a tissue rendered vulnerable, induced severe hemorrhagic necrosis in this tissue, provided either the antigen or the antibody was present in the blood stream at the time of the interaction. The antigens employed were animal proteins (*i.e.* blood sera of various animals) in experiments with actively and passively sensitized rabbits; and *Pneumococcus* Type III culture filtrates in the experiments with passively sensitized rabbits. The pneumococcus filtrates used were incapable of inducing the phenomenon by themselves.

The antibodies were actively and passively acquired. Active sensitization was induced by a single intravenous injection of the animal protein 7 days prior to the local injections. Shorter periods were inadequate. In passive sensitization experiments a small amount of antibody was found to be sufficient if it was injected locally and the antigen was given intravenously. When the antigen was injected locally and the antibody intravenously, large doses of the latter were required. In precipitation reactions, as pointed out previously (3), there were required larger amounts of antigen than antibody. Thus, human serum when diluted 1:1000 precipitated with undiluted anti-

Leptospira Medium.—After 24 to 48 hours, one observes a homogeneous, dense, sharply defined layer, extending downwards about 0.5 cm. from the surface. Below this is a slight, nebulous, uniform opacity, about 1 cm. high (Fig. 14). During the ensuing 3 or 4 days this deep layer becomes more dense and thereafter slowly extends to the bottom of the tube.

Gelatin.—The colonies are more mucoid and raised than on agar but are alike in other respects. In gelatin stab cultures there is an arachnoid growth along the line of inoculation but no liquefaction.

Broth.—A uniform turbid growth occurs with a slight, greyish sediment and without pellicle formation.

Litmus Milk.—Unchanged.

Potato.—Surface cultures show spreading, abundant growths having a light tan color.

Indol.—Not produced.

Nitrates.—Not reduced.

Carbohydrate Reactions.—Acid, but no gas, is produced in Hiss' serum water cultures containing dextrose, levulose, mannose, galactose, xylose, arabinose, and rhamnose. When the acid production is considerable, coagulation of the medium occurs and a pellicle is formed on the surface. Only a small amount of acid is produced with dextrin. An occasional strain slightly acidifies media containing either saccharose, lactose, inulin, or mannitol. Raffinose, salicin, dulcitol, amygdalin, maltose, trehalose, sorbitol, and inositol media are unchanged.

Oxygen Requirements.—The organism is aerobic and facultatively anaerobic.

Other Properties.—There is no characteristic odor in cultures. Heating for 30 minutes at 56°C. kills the organism; the optimum temperature for growth is, however, 28–30°C. Cultures are bile-resistant.

Serological Reactions.—Rabbit antiserum is regularly obtained in an agglutination titer of at least 1:1,000 for all strains of the bacterium. Such sera are specific: agglutination was noted neither with ordinary bacteria nor with 54 cultures of Gram-negative bacilli found in the conjunctival secretions or tissues of man or monkey. Although we met with three strains of morphologically and culturally similar organisms that were clumped, one in 1:10, the second in 1:100, and the third in 1:1,000 dilution of the serum, they were dissimilar in carbohydrate reactions. We are not now prepared to say whether these bacteria showed non-specific reactions or whether they were variants of the bacterium. Conversely, six rabbit antisera prepared with individual strains of the indifferent, Gram-negative organisms described above failed to agglutinate the special bacterium. Moreover, specific rabbit antisera yielded no cross-agglutination between this organism and *Bacterium granulosis*.

We should mention in this place that thus far cultures of the organism have not been agglutinated by the serum of monkeys having spontaneous or experimentally

has been discussed at length in previous communications (1, 3). The skin-preparatory factors, (*i.e.* those inducing the vulnerability) are bacterial products found only in certain bacterial culture filtrates. They are soluble, obtained under conditions of insignificant cell autolysis and produce either only a slight primary inflammation or no inflammation. The inflammatory property of bacterial filtrates is totally unrelated to these factors.

The reaction is severe and obtained shortly after the introduction of antigen and antibody. The hemorrhagic necrosis is somewhat similar in histological features to the Arthus phenomenon (4, 6). However, in control experiments horse serum was injected into two skin sites of rabbits which were sensitized by a single intravenous injection of horse serum 1 week previously. One skin site was prepared by an injection of *B. typhosus* filtrate 24 hours previously. The other skin site was unprepared. The prepared skin site showed severe hemorrhagic necrosis whilst the unprepared area was only slightly inflamed (Figs. 1 and 2). Thus, the injection of the antigen into a skin site prepared with a bacterial filtrate in a rabbit which had received a single sensitizing injection induced promptly hemorrhagic necrosis. In the Arthus phenomenon necrosis appears upon the injection of antigen into a rabbit which has been sensitized by six or seven injections of the protein in the course of several weeks (6).

Additional Comment

As seen from data presented in this and previous papers, tissues made vulnerable by bacteria or their soluble products undergo severe injury when acted upon by toxic principles resulting from intravascular antigen-antibody interaction. The interaction can be obtained in one of the following ways:

By separate intravenous injections of the antigen and the antibody; by intravenous injection of the antigen into an animal possessing actively acquired homologous antibodies; by injection of the antigen into the vulnerable area with a simultaneous intravenous injection of the antibody; and by injection of the antigen into the vulnerable area in rabbits possessing actively acquired antibodies. In the latter case, there apparently occurs intravascular production of the toxic principles at the site of the locally injected antigen through the interaction with the circulating antibodies.

Leptospira Medium.—After 24 to 48 hours, one observes a homogeneous, dense, sharply defined layer, extending downwards about 0.5 cm. from the surface. Below this is a slight, nebulous, uniform opacity, about 1 cm. high (Fig. 14). During the ensuing 3 or 4 days this deep layer becomes more dense and thereafter slowly extends to the bottom of the tube.

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We should mention in this place that thus far cultures of the organism have not been agglutinated by the serum of monkeys having spontaneous or experimentally induced folliculosis.

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EXPLANATION OF PLATE 56

FIG. 1. Section of a skin site injected with *B. typhosus* "agar washings" filtrate and 24 hours later with horse serum in a rabbit sensitized by a single intravenous injection of horse serum 1 week previously. Skin removed 5 hours after the second intradermal injection. Note necrobiosis, rupture of blood vessel wall and severe hemorrhage. Hematoxylin and eosin. $\times 160$.

FIG. 2. Section of a skin site injected with streptococcus culture filtrate and 24 hours later with horse serum in a rabbit sensitized by a single intravenous injection of horse serum 1 week previously. Skin removed 5 hours after the second intradermal injection. Note slight inflammation. Hematoxylin and eosin. $\times 320$.

FIG. 3. Section of a skin area injected with horse serum in a rabbit sensitized by a single intravenous injection of horse serum 1 week previously. Skin removed 5 hours after the intradermal injection of horse serum. Note slight inflammation. Hematoxylin and eosin. $\times 320$.

Leptospira Medium.—After 24 to 48 hours, one observes a homogeneous, dense, sharply defined layer, extending downwards about 0.5 cm. from the surface. Below this is a slight, nebulous, uniform opacity, about 1 cm. high (Fig. 14). During the ensuing 3 or 4 days this deep layer becomes more dense and thereafter slowly extends to the bottom of the tube.

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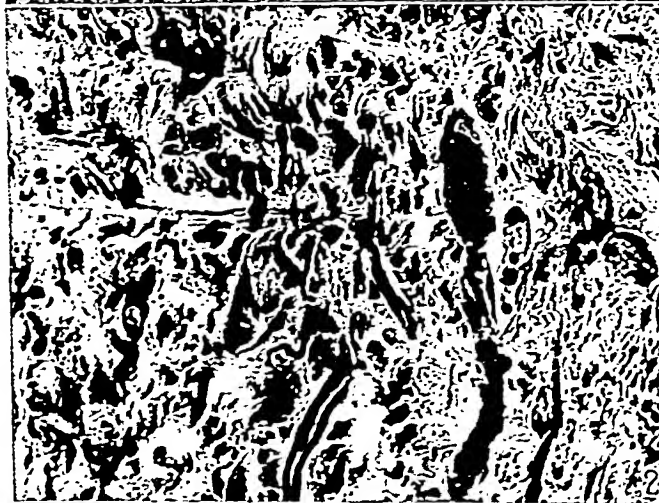
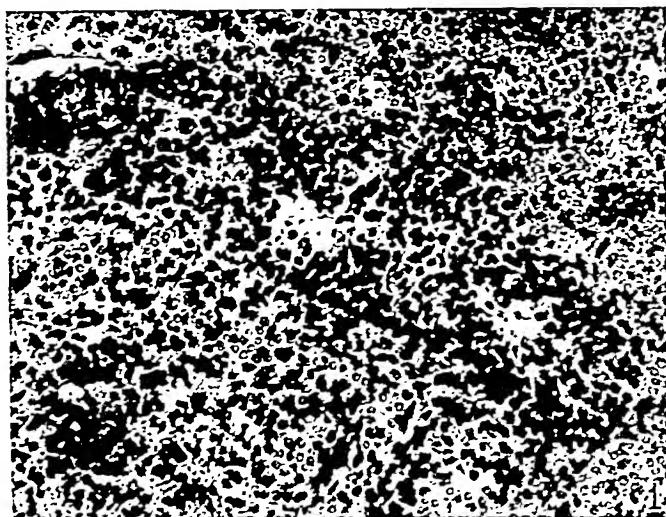
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accompanied by hemorrhage. This stage lasted for about a week or two, and was followed by the familiar, second period of inflammatory, progressive follicular conjunctivitis, during which time the uninoculated conjunctivae also became involved.

In the appended drawings is represented the clinical appearance of a *rhesus* monkey having spontaneous folliculosis (Fig. 20 A), showing also the characteristic follicles on the bulbar conjunctiva (Fig. 20 B). The figures can be used as a standard for comparison of these clinical appearances with those produced by inoculation of cultures (Fig. 20 C); the similarity of the natural and the induced disease is clearly shown.

The histopathology of experimental folliculosis following injection of the organism was also similar to that of the spontaneous disease, a description of which has already been given (1) (Figs. 15 to 19). Studies of stained sections of conjunctival tissue, derived from either the natural or the induced disease, revealed discrete or clumped, Gram-negative bacilli having morphological resemblances to *Bacterium granulosis* (Figs. 8 and 9).

The numerical results of the tests on pathogenicity of the seven cultures of *Bacterium simiae* recovered from stock monkeys having spontaneous folliculosis are tabulated.

Strain No.	No. of <i>rhesus</i> monkeys inoculated	No. of <i>rhesus</i> monkeys showing positive reactions
5A2	6	5
29B	2	2
97B	2	1
10A	2	1
50B	1	1
601	2	2
605	6	3
Totals..... 7	21	15

In addition, a chimpanzee that also received Strain 5A2 developed folliculosis indistinguishable from the spontaneous or the experimental disease of monkeys. Also, tissues taken from monkeys successfully inoculated with the cultures produced in the conjunctivae of fresh *rhesus* monkeys the characteristic follicular reaction.

Conjunctival Swabbing.—The method consists of applying to a cotton swab a suspension of the organism, prepared by adding 2 cc. of saline

STUDIES ON THE ETIOLOGY OF SPONTANEOUS CONJUNCTIVAL FOLLICULOSIS OF MONKEYS

II. BACTERIOLOGICAL EXPERIMENTS

By PETER K. OLITSKY, M.D., JEROME T. SYVERTON, M.D., AND
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(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATES 57 TO 59

(Received for publication, January 21, 1933)

In the first paper of this series (1), we described the clinical appearance and histopathology of the inflammatory type (Type II) of spontaneous conjunctival folliculosis as it occurs in *Macacus rhesus* monkeys. It was shown that the incitant of the affection is apparently a specific infectious agent of microbic rather than ultramicroscopic type. In this paper we shall record the results of bacteriological studies on the disease.

Methods

The methods employed by Noguchi in his trachoma investigations (2) were closely followed. For cultivation tests, however, we depended on leptospira medium and horse blood agar plates and slants. Since the following modifications of Noguchi's original media not only facilitate the recovery of *Bacterium granulosis* (2) from affected tissues but also result in more profuse cultural growth, we employed them in the present study.

In preparing leptospira medium, 0.2 instead of 0.1 part of laked rabbit erythrocytes was added, and the horse blood agar was changed from its original composition to:

Defibrinated horse blood.....	60
2 per cent nutrient agar, as employed by Noguchi.....	500
Mixture of carbohydrates (dextrose 10 per cent, sucrose 10 per cent, mannose 2.5 per cent, levulose 2.5 per cent, and inulin 2.5 per cent, sterilized by filtration through Berkefeld V or N candles).....	25

It should be emphasized that both media should be freshly prepared; the agar slants should contain 0.5 to 1.5 cc. of condensation water and the horse blood and rabbit serum should be obtained a short time only before use.

Thus the simian organism was recovered not only from stock *rhesus* monkeys having spontaneous folliculosis but also from monkeys and an ape experimentally infected with this bacterium. Indeed, these recovered strains were again proved to be specifically active in fresh *rhesus* monkeys and chimpanzees.

To sum up the total number of positive reactions obtained in monkeys and apes with cultures of the bacterium, irrespective of their source, that is whether recovered from stock *rhesus* monkeys with folliculosis or from animals experimentally infected by means of cultures, we find that of thirty-three monkeys and four chimpanzees inoculated, twenty-four of the former and all of the latter showed experimental follicular conjunctivitis.

Duration of Pathogenicity of Cultures.—One strain was found to be pathogenic for monkeys up to 355 days after its isolation; thereafter it proved to be inactive. The experimental disease was induced with another culture on the 21st day after its isolation but not on the 170th day. Hence it appears that the duration of pathogenicity of different strains maintained on artificial media can vary and with time lose the power to infect. Furthermore, infective action of the microorganism was observed in its first to tenth subplant in media, subcultures more remote than the second having been made at about a month's interval.

DISCUSSION AND CONCLUSIONS

In the bacteriological study here reported, we undertook an investigation of the flora associated with spontaneous conjunctival folliculosis. Following the plan of Noguchi (2), monkeys and chimpanzees were inoculated with the different organisms recovered from affected tissues. By this means, we disclosed among the bacteria a new species, *Bacterium simiae*, which was found to be specifically active, in that it induced follicular reactions in the conjunctiva apparently indistinguishable from the disease as it occurs in nature.

The specific action of the bacterium in animals is the more striking when it is compared with the innocuousness of other organisms isolated from cases of folliculosis, and also when considered in relation to the behavior of quarantined animals. While the disease arises spontaneously in stock animals, of some 300 normal *rhesus* monkeys—these being isolated in lots of ten to twenty and quarantined from 6 to

Description of the Microorganism

The bacterium can be defined as a minute, Gram-negative, aerobic bacillus which is monotrichous, encapsulated, and actively motile. A detailed description of its morphological and growth characteristics follows.

Morphology.—The organisms occur as slender, tenuous bacilli, frequently discrete, less often in short chains or in parallel arrangement of two or three members, and least often as diplobacilli. They measure in width 0.2 or 0.3 μ and in length from 0.8 to 1.2 μ ; the shorter forms are found in leptospira medium cultures, the longer ones in agar media. They have pointed ends and each organism is surrounded by a capsule which is somewhat wider than the somatic material itself. The capsular material is often demonstrable by ordinary staining methods. A single polar flagellum appears to be attached to the capsule but not to the bacterial body.¹ In rare instances the flagellum is double and infrequently its capsular attachment is lateral. The bacilli are actively motile; the motility being influenced neither by the age of the culture nor the particular medium used. They usually dart zigzag across a field but in some instances move in a pivotal or whirling manner, without forward progression.

Staining Reactions.—The organisms are invariably Gram-negative, whether in film preparations of cultures or in stained sections of affected tissues; they are not acid-fast. Spores or metachromatic granules are not detectable with Neisser's, toluidin blue, or Giemsa's stains. Infrequently, Loeffler's alkaline methylene blue solution stains certain parts of the bacterial protoplasm more deeply. In dark-field examinations, however, no spores or granules can be seen. (Figs. 1 to 9; Fig. 4 shows a characteristic appearance of ordinary Gram's stain film preparations,—a blurred background consisting of debris of capsular and flagellar material in which are embedded the bacterial bodies.)

Agar Plates.—The colonies appear on plain agar as small, circular, greyish, translucent growths. They are also smooth, convex, and slightly raised, tending toward confluence and having a sticky, or mucoid, consistence. Their appearance is similar on blood agar except that the colonies, more highly translucent and colorless in early growths, become greyish after 2 or 3 days (Fig. 10). We wish to emphasize that a positive culture of affected tissue shows at best only one, two, or three colonies of the microorganism scattered among a number of other miscellaneous growths (Fig. 11). Hence it is essential to use the same inoculum for several plate cultures.

Agar Slants.—The growth on this medium reveals a greyish white to white, moist, mucoid, heaped up, glistening appearance. When blood is added to the medium, the growth is more profuse (Figs. 12 and 13).

¹ The apparent attachment of flagella to capsules was first shown by Churchman, J. W., and Emelianoff, N. V., *Proc. Soc. Exp. Biol. and Med.*, 1932, 29, 996.

monkey having spontaneous folliculosis. To be noted at arrow-head, a clump of the bacteria. $\times 1,500$.

FIGS. 10 to 14. These figures represent cultural characteristics.

FIG. 10. 48 hour growth; colonies on blood agar plate. $\times 10$.

FIG. 11. 48 hour growth; same medium; showing a mixed culture derived from a tissue suspension. Only two colonies of the bacterium are visible, indicated by arrow-heads. Natural size.

FIG. 12. 48 hour growth; plain agar slant. Natural size.

FIG. 13. 48 hour growth; blood agar slant. Natural size.

FIG. 14. 48 hour growth; leptospira medium. Natural size.

PLATE 58

FIG. 15. Section of conjunctiva of a monkey having spontaneous folliculosis. Hematoxylin and eosin stain. Three follicles, one of them deeply situated, and the destruction of the superficial epithelium, are shown. $\times 122$.

FIG. 16. Section of same magnified $\times 1,000$. The zonal lymphoid cell layer (L), within which is the core of macrophages (M), is represented.

FIG. 17. Section of conjunctiva of a monkey having experimental folliculosis induced by culture injection. Hematoxylin and eosin stain. To be noted is the similarity of lesions with those represented in Fig. 15. $\times 122$.

FIG. 18. Section of conjunctiva of another monkey with experimental folliculosis following culture injection. Hematoxylin and eosin stain. A prodigious follicle is shown, which may have resulted from the confluence of two or three contiguous lesions. $\times 122$.

FIG. 19. Same as Fig. 17, but magnified $\times 1,000$. Here can be seen microscopic changes similar to those found in the spontaneous disease (Fig. 16).

PLATE 59

FIG. 20. All drawings about twice natural size.

A. Eye of *M. rhesus* monkey having spontaneous folliculosis. Fully developed case, of over 5 months' duration. The chief features are the large discrete and massed succulent follicles, also seen over the tarsal plate, the edema and congestion, and the invisibility of vascular structures.

B. The bulbar conjunctiva of the same case. To be noted are the characteristic, discrete follicles.

C. Eye of *M. rhesus* monkey having experimental folliculosis induced by subconjunctival inoculation of the simian organism in the left upper lid. Fully developed reaction, 6 months after inoculation. There is no distinction between this clinical appearance and that of spontaneous folliculosis occurring in nature (cf. A). The bulbar conjunctiva of this animal revealed follicles similar to those shown in B.

An examination of the characteristics of the microorganism reveals that it has a generic resemblance to *Bacterium granulosis* but differs from it in certain specific properties; namely, in motility, in manner of growth on agar or in leptospira medium, in distinctive carbohydrate reactions, and finally, in serological specificity. Hence to designate the organism we have named it tentatively *Bacterium simiae*, n. sp.²

Spontaneous Conjunctival Folliculosis in Chimpanzees

We have heretofore described the spontaneous follicular conjunctivitis as it occurs in *Macacus rhesus* monkeys. Further observations have shown that a similar disease exists among chimpanzees. In two lots, one containing eight and the other ten apes, the affection was present in two animals of the first group and in three of the second. We were able to study bacteriologically only three of the affected chimpanzees, and tarsectomized tissue was not available—only conjunctival scrapings and follicular contents. It has already been indicated that such material is inadequate for cultural purposes. However this may be, the simian organism was not recovered from the three apes, but as will be shown later, we have been more successful in implanting in chimpanzees, by means of cultures of the *rhesus* organism, a disease similar to that found in monkeys.

Pathogenicity Experiments

*Subconjunctival Inoculation.*³—*Macacus rhesus* monkeys and a chimpanzee were inoculated in the upper conjunctiva of one eye with each of the seven strains of the simian organisms that were cultivated from stock *rhesus* animals having spontaneous folliculosis.

The result of the inoculation was a counterpart of that following the injection of suspensions of tissue derived from monkeys having the disease, as described in detail in the preceding paper of this series (1).

There was, however, one difference to be noted. The immediate effects following injection of cultures consisted in more conspicuous inflammatory reaction of the skin and conjunctiva of the eyelid, as evidenced by more intense edema and swelling of the structures, ac-

² The generic name is based on the *Ehrenberg emend. Jensen*, classification of Bacteriaceae; the specific name is derived from *simia* (monkey or ape).

³ Ether anesthesia was employed in all operations on animals.

monkey having spontaneous folliculosis. To be noted at arrow-head, a clump of the bacteria. $\times 1,500$.

FIGS. 10 to 14. These figures represent cultural characteristics.

FIG. 10. 48 hour growth; colonies on blood agar plate. $\times 10$.

FIG. 11. 48 hour growth; same medium; showing a mixed culture derived from a tissue suspension. Only two colonies of the bacterium are visible, indicated by arrow-heads. Natural size.

FIG. 12. 48 hour growth; plain agar slant. Natural size.

FIG. 13. 48 hour growth; blood agar slant. Natural size.

FIG. 14. 48 hour growth; leptospira medium. Natural size.

PLATE 58

FIG. 15. Section of conjunctiva of a monkey having spontaneous folliculosis. Hematoxylin and eosin stain. Three follicles, one of them deeply situated, and the destruction of the superficial epithelium, are shown. $\times 122$.

FIG. 16. Section of same magnified $\times 1,000$. The zonal lymphoid cell layer (L), within which is the core of macrophages (M), is represented.

FIG. 17. Section of conjunctiva of a monkey having experimental folliculosis induced by culture injection. Hematoxylin and eosin stain. To be noted is the similarity of lesions with those represented in Fig. 15. $\times 122$.

FIG. 18. Section of conjunctiva of another monkey with experimental folliculosis following culture injection. Hematoxylin and eosin stain. A prodigious follicle is shown, which may have resulted from the confluence of two or three contiguous lesions. $\times 122$.

FIG. 19. Same as Fig. 17, but magnified $\times 1,000$. Here can be seen microscopic changes similar to those found in the spontaneous disease (Fig. 16).

PLATE 59

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Pathogenicity Experiments

*Subconjunctival Inoculation.*³—*Macacus rhesus* monkeys and a chimpanzee were inoculated in the upper conjunctiva of one eye with each of the seven strains of the simian organisms that were cultivated from black *rhesus* animals having spontaneous folliculosis.

The result of the inoculation was a counterpart of that following the injection of suspensions of tissue derived from monkeys having the disease, as described in detail in the preceding paper of this series (1). There was, however, one difference to be noted. The immediate effects following injection of cultures consisted in more conspicuous inflammatory reaction of the skin and conjunctiva of the eyelid, as evidenced by more intense edema and swelling of the structures, ac-

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solution to an agar slant growth, and then gently rubbing the material on the conjunctiva of one eye of a monkey. This was done daily for 6 or 7 consecutive days.

Four *rhesus* monkeys were swabbed in this manner. Two of them became affected, showing the first signs of the experimental disease 3 days after the last swabbing. 7 days later the untreated eye developed folliculosis.

To summarize, the seven strains of the simian organism recovered from stock *rhesus* monkeys having the spontaneous disease, in each instance induced characteristic folliculosis, positive reactions having been obtained after subconjunctival inoculation or swabbing, in seventeen of twenty-three *rhesus* monkeys and in a single chimpanzee.⁴

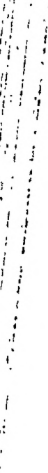
Recovery of Cultures from Experimental Folliculosis

We attempted to recover the organism from four *rhesus* monkeys and the ape which had developed characteristic experimental folliculosis as a result of inoculation of *Bacterium simiae*. Of these five attempts, three were successful: two cultures being derived from the affected *rhesus* monkeys, and one from the chimpanzee. The successful isolations were obtained from tissues 18 days to 11 weeks after inoculation.

The recovered microorganisms were again introduced into ten *rhesus* monkeys and three chimpanzees with these results:

Strain No.	Source	No. of animals inoculated	No. of animals showing positive reactions
57B	<i>Rhesus</i> monkey experimentally infected with culture	5 <i>rhesus</i> monkeys	4 monkeys
140B	<i>Rhesus</i> monkey experimentally infected with culture	{ 2 <i>rhesus</i> monkeys 2 chimpanzees	1 monkey 2 chimpanzees
V	Chimpanzee experimentally infected with culture	3 <i>rhesus</i> monkeys 1 chimpanzee	2 monkeys 1 chimpanzee

⁴ While the disease arising spontaneously (1) or following the inoculation of cultures of the microorganism is, as a rule, characterized by slow progression and by persistence, lasting usually throughout the life of the animal, we have observed an occasional monkey recover. We may therefore suppose that the resistance to inoculation as shown by some of the animals is due to their complete recovery and consequent immunity.



14 weeks—not one has as yet shown folliculosis. With the insignificant exception already mentioned (1), the experimental disease was produced only when the inoculum contained either folliculosis tissue or cultures of the simian organism.

Apart from these findings, the experimental results indicate that (a) the bacterium has thus far been recovered only from folliculosis cases and not from other forms of conjunctivitis nor from normal tissues; (b) the microorganism has been isolated not only from affected conjunctivae of stock monkeys but also from the tissue of animals—macaques and apes—experimentally infected with the bacterium, and (c) such recovered cultures have, in turn, been found to be specifically pathogenic in normal *rhesus* monkeys and chimpanzees.

We may therefore postulate from this experimental study that an intimate relation exists between *Bacterium simiae* and spontaneous conjunctival folliculosis of simians.

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2. Noguchi, H., *J. Exp. Med.*, 1928, 48, suppl. 2, 1-53.

EXPLANATION OF PLATES

PLATE 57

FIGS. 1 to 9. Morphological appearances of *Bacterium simiae*.

FIG. 1. Gram's stain. *Leptospira* medium culture. $\times 1,000$.

FIG. 2. Gram's stain. *Leptospira* medium culture. $\times 1,500$.

FIG. 3. Methylene blue stain. Film preparation from an agar plate; such preparations usually reveal longer forms than those seen in *leptospira* medium cultures. $\times 1,800$.

In the above three figures incomplete or faint capsules can be observed, although ordinary stains have been employed.

FIG. 4. Gram's stain. Showing the background of capsular and flagellar debris—the usual, characteristic appearance in film preparations. $\times 1,000$.

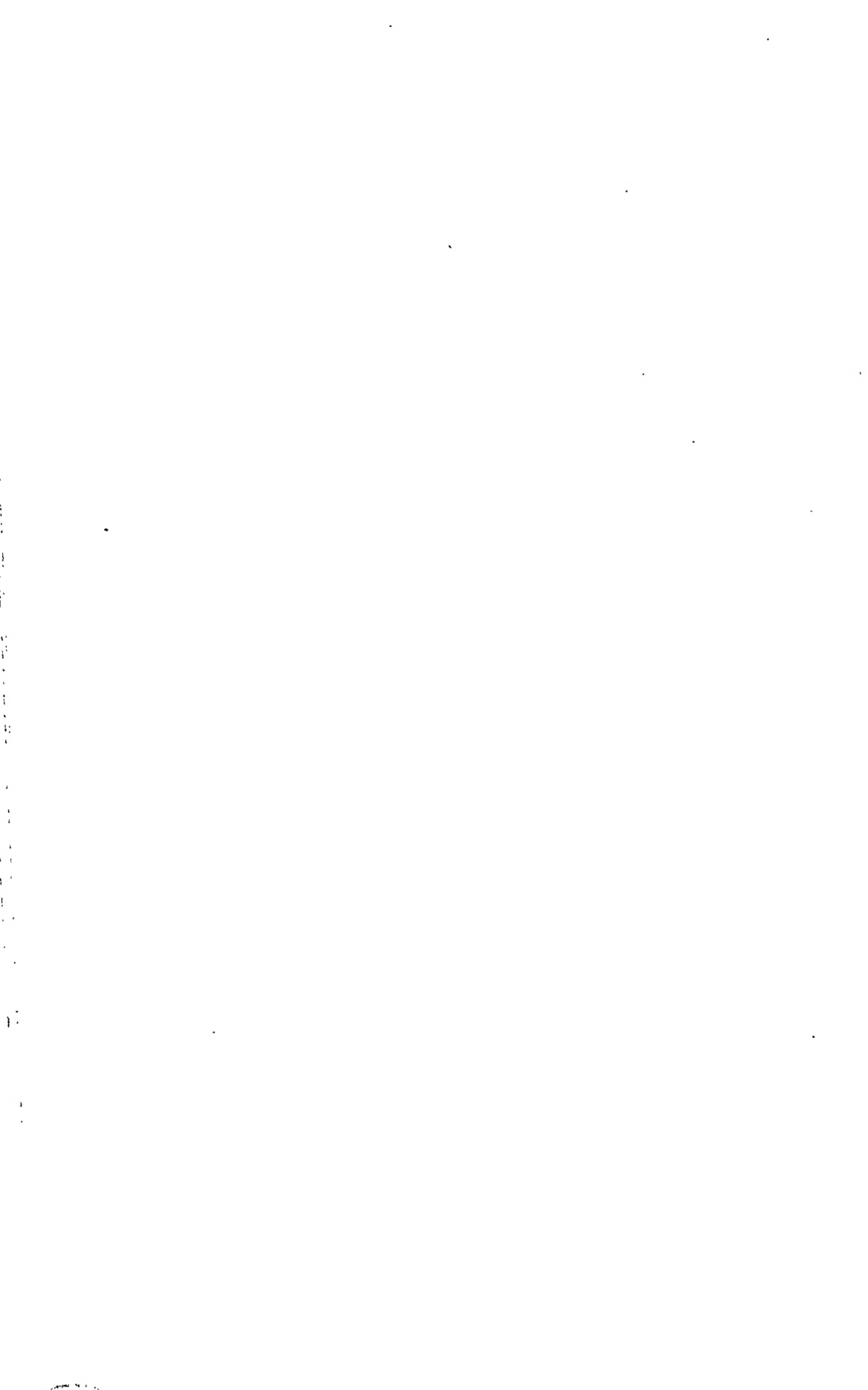
FIG. 5. Capsule and flagellum. Casares-Gil stain. $\times 2,000$.

FIG. 6. Same. Depicting a longer and broader flagellum. $\times 1,800$.

FIG. 7. Same. Illustrating two organisms, each with its separate capsule and flagellum. $\times 2,000$.

FIG. 8. Eosin-methylene blue stain. Conjunctival tissue derived from monkey injected with a culture of the bacterium. At arrow-head can be seen discrete organisms. $\times 1,000$.

FIG. 9. Eosin-methylene blue stain. Conjunctival tissue removed from a

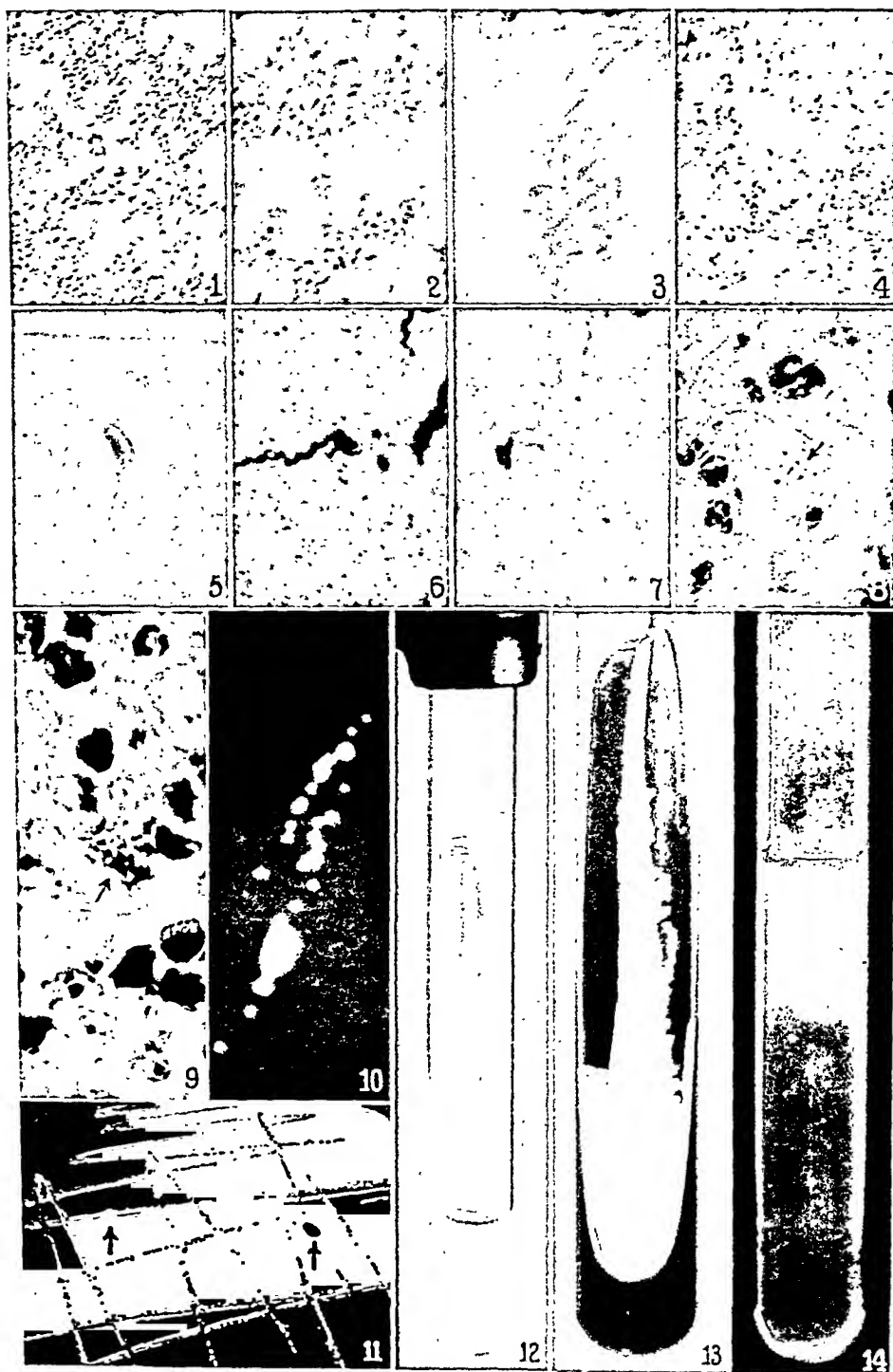




Photographed by Louis Schmidt

Price-Jones (4) rendered rabbits anemic by bleeding and by injecting phenylhydrazine, and examined smears of the bone marrow from animals killed in different stages and degrees of anemia. He found an increase in the number of nucleated cells over the normal more or less proportional to the degree of anemia. A marked erythroblastic reaction in the bone marrow, associated with the blood changes, has been noted by Muir and McNee (5) in rabbits made anemic by hemolytic serum. McMaster and Haessler (6) have reported that in rabbits repeatedly bled and reinjected with more than an equivalent amount of hemoglobin, the anemia was accompanied by an extensive spread of the red marrow with an increase in the size and abundance of the erythropoietic islands. Marrows of animals which were merely bled did not undergo these changes. Muller (7) has studied the effects of various colloidal substances on the blood and bone marrow. Small doses of collargol injected into rabbits produced erythrocytic marrow hyperplasia; larger doses finally produced an aplastic marrow with anemia. Injections of Weber's India ink gave rise to a persistent high degree of normoblastosis in the peripheral blood, associated with hyperplasia of the erythroid cell series in the marrow. Injections of gum shellac produced normoblastosis, erythroblastosis, and some degree of polycythemia in the circulating blood, with intense erythrocytic hyperplasia in the marrow. Robertson (8) found a decrease in reticulocytes in the marrow of rabbits rendered plethoric by transfusion, indicating a retardation of erythropoiesis. A severe but transient anemia occurred when the transfusions were stopped, the anemia being the result of a relatively inactive marrow. Sabin and Doan (1) have found that in normal rabbits the major supply of nucleated red cells is at the more mature stages (normoblasts 69 per cent, early and late erythroblasts 30 per cent, and megaloblasts 0.01 to 0.04 per cent); and Sabin (2) states that only in extreme anemia is the marrow thrown back to megaloblasts and endothelium.

Further experiments involving different methods of studying the relation between the erythroid cells of the marrow and the peripheral blood picture would be valuable. During recent years the number of young erythrocytes in the circulating blood, as determined by reticulocyte counts, has been recognized as a reliable index of erythrogenic activity in the bone marrow. By means of such reticulocyte counts it is possible to follow the level of red blood cell production in experimental animals with a reasonable degree of accuracy. The present investigation was designed to correlate the peripheral blood picture, especially the reticulocyte counts, with the pattern of the erythroid cells in the bone marrow. This study was made in normal animals and in animals in which blood production was increased by acute and chronic hemorrhage and by red blood cell destruction due to acetyl phenylhydrazine.



ERYTHROID CELLS IN BONE MARROW

Control Animals

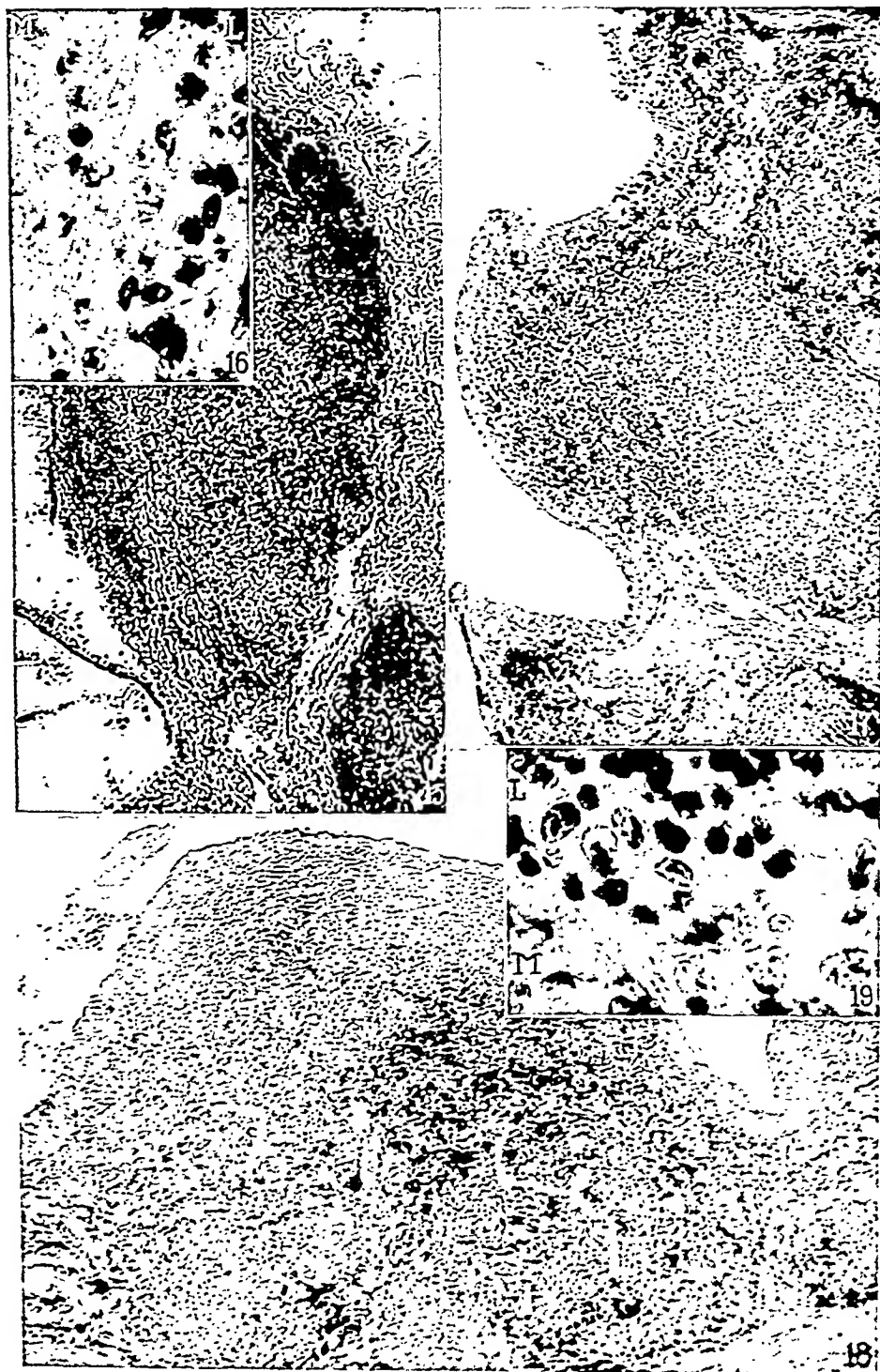
The peripheral blood of six rabbits was examined frequently over a period of several days to establish normal blood values. Immediately after the final blood examination the animals were sacrificed and the bone marrow was studied. Condensed protocols are shown in Table I. The data on the peripheral blood of these animals compare favorably with the values given for normal rabbits by Scarborough (12). Although the average red blood cell count was high (5.14 million per c.mm.) there was a constantly maintained reticulocyte count which

TABLE I
Peripheral Blood Values and Percentages of Erythroid Cells in the Bone Marrow of Six Normal Rabbits

Rabbit No.	Peripheral blood values (average of all counts)			Peripheral blood values (immediately before death)			Erythroid cells in the bone marrow			
	R.B.C. per c.mm.	Hb	Absolute reticu- locytes per c.mm.	R.B.C. per c.mm.	Hb	Ab- solute reticu- locytes per c.mm.	Ery- thro- blasts and megaloblasts	Normo- blasts	Reticu- locytes	Mature erythro- cytes
	millions	per cent	millions	millions	per cent	millions	per cent	per cent	per cent	per cent
1	5.23	72	0.14	5.38	75	0.14	6.3	16.3	28.2	49.2
2	4.80	75	0.12	5.00	72	0.16	5.8	15.8	24.8	53.6
3	5.13	71	0.09	5.16	74	0.10	7.3	17.2	29.6	45.9
4	5.24	62	0.12	5.40	62	0.14	7.1	19.2	25.3	48.4
5	5.37	65	0.13	5.51	69	0.11	7.5	24.1	31.3	37.1
6	5.09	66	0.14	5.11	67	0.17	5.2	21.4	30.5	42.9
Average ..	5.14	69	0.12	5.26	70	0.14	6.5	19.0	28.3	46.2

averaged 0.12 million per c.mm. The red blood cells usually showed a mild but distinct degree of anisocytosis. Nucleated red blood cells were never seen in the peripheral blood smears.

The differential counts of the erythroid cells in the femoral bone marrow of these six animals were relatively uniform (Table I). The average of the six counts revealed that out of the total number of erythroid cells nearly one-half were mature and over one-fourth were reticulocytes, indicating that three-fourths of the erythroid cells were ready for immediate delivery into the blood stream. Only one-fourth of the cells were in the less mature nucleated stages of development.



hours apart, 26 to 50 per cent of the blood volume was removed. The animals were sacrificed at or near the peak of the reticulocyte response and the marrows examined. Condensed protocols are shown in Table II.

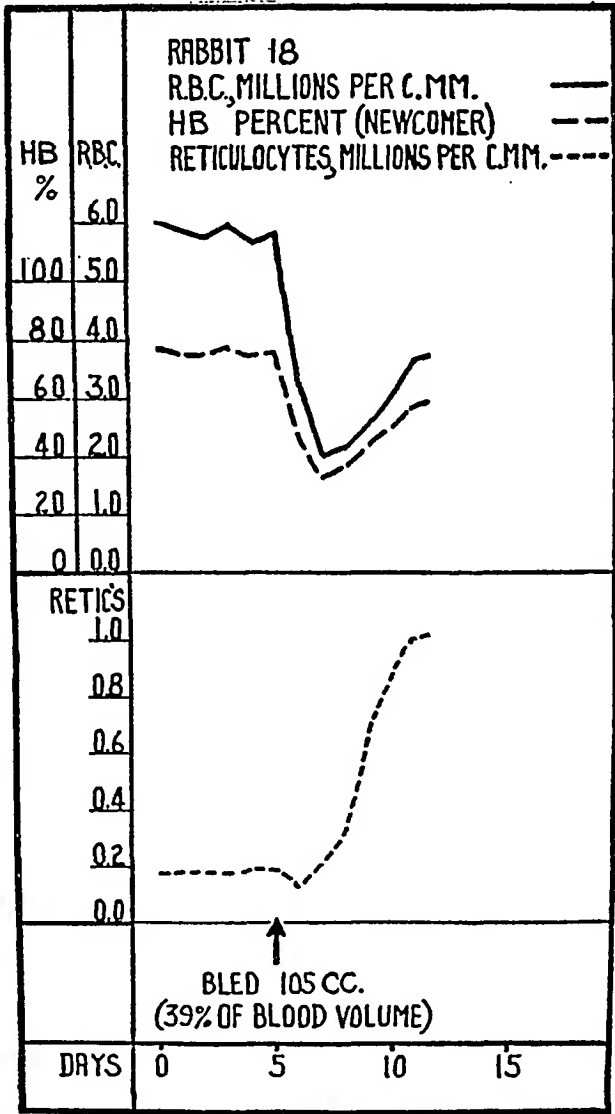
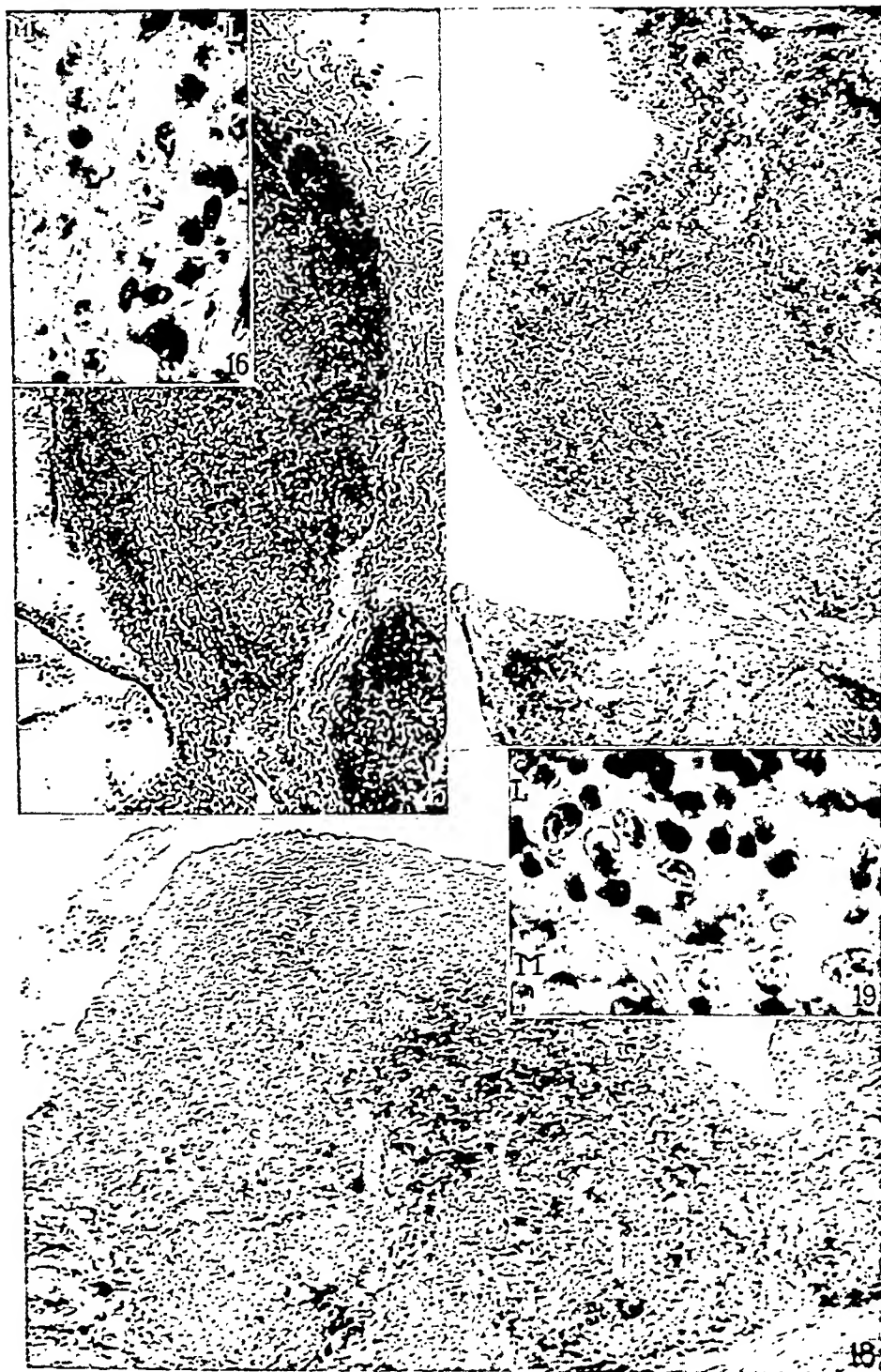


CHART 1. Observations of the blood in Rabbit 18 subjected to acute hemorrhage.

Chart 1 shows the typical course of blood values in an animal subjected to acute hemorrhage. Although an average of only 38 per cent



ERYTHROID CELLS IN BONE MARROW

The Findings in Chronic Hemorrhage

After a control period for determination of normal peripheral blood values, the four animals included in this series were subjected to a long course of bleedings. The animals were sacrificed 24 to 36 hours after the last bleeding and the marrows then examined. The condensed protocols are shown in Table III.

TABLE III
Peripheral Blood Values, Bleeding Data, and Marrow Findings in Rabbits Subjected to Chronic Hemorrhage

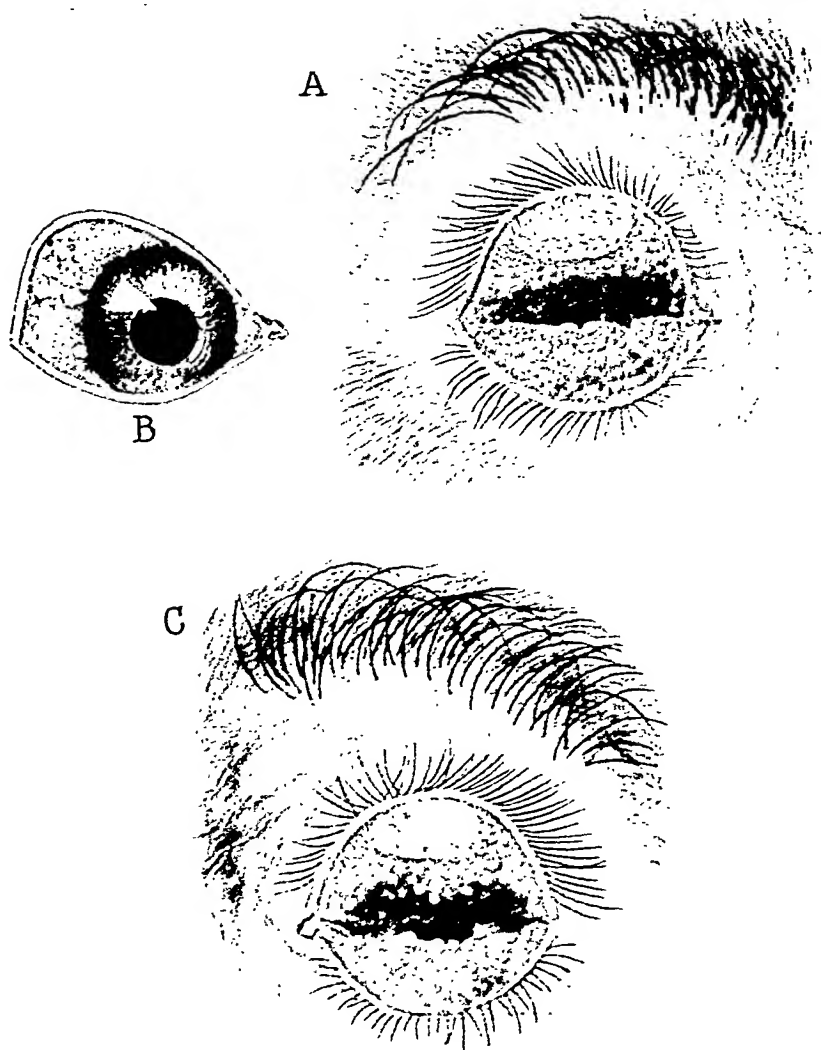
Bleeding Data, and Marrow Findings in Rabbits Subjected to Chronic Hemorrhage

Rabbit No.	Average of peripheral blood values before bleeding			Bleeding data					Average of peripheral blood values during period of bleeding			Peripheral blood values immediately before death			Erythroid cells in bone marrow			
	R.B.C. per c.mm.		Absolute No. reticulocytes per c.mm.	No. of bleedings	Duration of bleeding period	Total cc. of blood removed	Total per cent of blood volume removed	Hb removed	R.B.C. per c.mm.		Absolute No. reticulocytes per c.mm.	R.B.C. per c.mm.		Absolute No. reticulocytes per c.mm.	Erythroblasts and megakoblasts	Normoblasts	Reticulocytes	Mature erythrocytes
	mil-lions	per cent							mil-lions	per cent	mil-lions	mil-lions	per cent	mil-lions	per cent	per cent	per cent	per cent
10	5.17	69	0.15	18	122	654	240	65	4.38	57	0.43	3.24	41	0.52	19.5	42.3	23.5	14.7
15	6.38	76	0.13	24	72	730	214	72	4.73	62	0.58	3.73	55	0.79	12.5	45.4	29.5	12.6
17	6.10	70	0.12	21	53	640	200	59	4.37	54	0.58	3.84	48	0.77	9.3	39.4	39.5	11.8
24	5.13	63	0.13	18	40	480	204	36	3.62	46	0.51	3.61	47	0.65	9.8	41.2	26.5	22.5
Average.	5.69	70	0.13	20	72	626	215	58	4.28	55	0.53	3.61	48	0.68	12.8	42.1	29.7	15.4

Measured amounts of blood were sufficient to produce

Measured amounts of blood were removed frequently in quantities sufficient to produce a sustained reticulocytosis without giving rise to a severe anemia. A total of 200 to 240 per cent (average 215 per cent) of the blood volume was removed; but, as suggested before, the blood volume values are possibly too high. Chart 2 shows the course of the peripheral blood values and the bleedings in Rabbit 15, which is typical of the series as a whole.

During the period of bleeding the average reticulocyte count was 0.53 million per c.mm., or approximately 4 times as high as the pre-



Joeris^c Schmidt¹⁷-32- 20

Differential counts of erythroid cells in the marrow showed a reversal of the normal ratios between mature erythrocytes, reticulocytes, and normoblasts, being similar in this respect to the marrows of acute hemorrhage. Normoblasts were again the predominant type of cell. The percentage of reticulocytes was not appreciably changed from the normal; mature erythrocytes were much reduced. However, the most significant feature of the differential counts was the increase of erythroblasts and megaloblasts to nearly double the normal percentage. The rise in percentage of this group of cells was chiefly due to an increase in the numbers of late erythroblasts. Megaloblasts were present in small numbers.

The marrows of these animals were distinctly hyperplastic. The red marrow was soft and exceedingly friable, filling the entire shaft of the femur and the proximal half of the tibia. The distal half of the tibia contained fatty marrow, and a few small flecks of gross fat were seen in the distal fourth of the red femoral marrow. Sections showed the presence of fat cells in all but a few scattered areas of the red marrow, but these fat cells were greatly reduced in size and number. Erythropoietic islands were seen to be large and numerous, and, in many areas, confluent. The spleens of these animals were normal in the gross, but two of them showed some slight evidence of early extramedullary hematopoiesis.

The Findings in Phenylhydrazine Anemia

Following a period of several days, during which the normal blood values were determined, four animals were injected with acetyl phenylhydrazine to induce intracorporeal red blood cell destruction. Small doses of the drug were injected subcutaneously every 2nd or 3rd day in amounts sufficient to produce a sustained reticulocytosis. The animals were sacrificed and the marrows examined 24 hours after the last injection. Protocols are shown in condensed form in Table IV.

Chart 3 shows the course of the blood findings in Rabbit 19. This animal was given a relatively large initial dose of acetyl phenylhydrazine and responded with a high reticulocyte rise followed by an extensive drop in reticulocytes. Although this same phenomenon of abrupt rise and fall was noted in all four animals, those in which the

THE EFFECTS OF BLOOD LOSS AND BLOOD DESTRUCTION UPON THE ERYTHROID CELLS IN THE BONE MARROW OF RABBITS*

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Department of Medicine, Indiana University School of Medicine, Indianapolis)*

(Received for publication, November 29, 1932)

The consideration of the bone marrow as a major organ of the body has recently been urged by Sabin and Doan (1-3). Although the component parts of this organ are widely scattered, and connected only indirectly through the blood stream, these parts tend to function as a unit. A continuous state of physiologic activity is maintained under normal conditions, reciprocal relationships existing between the various types of erythroid and myeloid cells of the circulating blood and their precursors in the marrow. The bone marrow also possesses a reserve power which is utilized in times of stress to produce more than the normal requirements of blood cells. Doan (3) pictures this reserve as existing in two distinct forms: first, the actual limited numbers of more mature cells ready for delivery into the blood stream, and second, the growth possibilities of the immature cells. He further states that a "shift to the left" or to more immature types of cells is the response to a demand for increased blood production.

Many investigators have been interested by the problem of erythroid marrow reserve, and numerous experiments have been done to determine the changes occurring in the marrow cells when the reserve power is activated. A productive organ such as the bone marrow can usually be studied most advantageously in relation to its product. Hence much of the experimental work on marrow reserve has involved the relationship existing between the pattern of the erythroid cells of the marrow and the peripheral blood picture.

* This study was made possible by funds supplied by the Henry Strong Denison Medical Foundation.

hydrazine, the red blood cells were destroyed within the body, thus retaining their content. In the latter weeks of the injection period

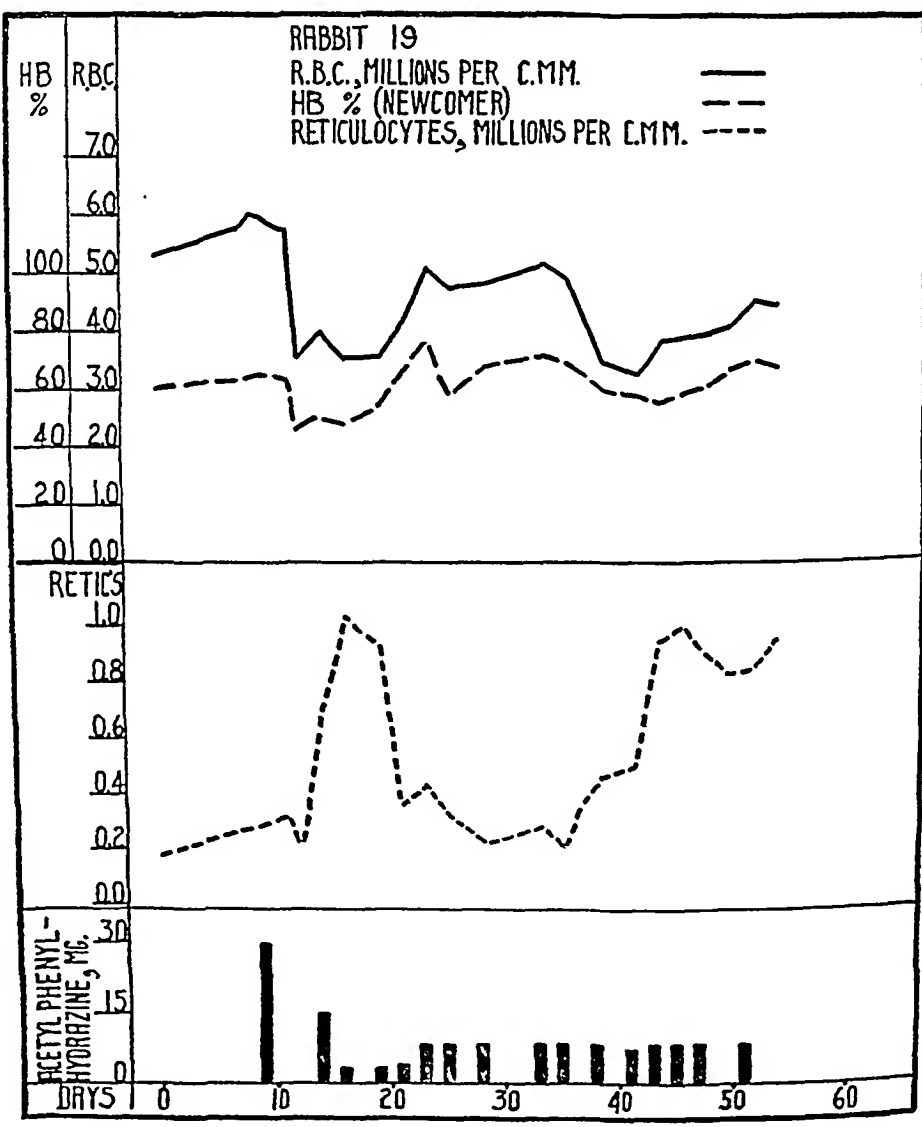


CHART 3. Observations on the blood of Rabbit 19 injected with acetyl phenylhydrazine.

the red blood cells showed a high degree of anisocytosis. Normoblasts were seen in a majority of the peripheral blood smears, but always in small numbers.

Methods

Healthy rabbits from standard stock were used in the experiments. Animals which showed evidences of infection or other abnormalities were discarded and the data were not included in the results. The animals were fed an adequate and highly nutritious diet and showed some gain in weight when observed for a period of several weeks.

At frequent intervals, usually daily or every other day, the peripheral blood obtained by puncture of an ear vein was examined. This included red blood cell count, hemoglobin determination, and reticulocyte count on a smear stained with brilliant cresyl blue and counterstained with Wright's stain. The absolute number of reticulocytes was calculated in each case and recorded in millions per cubic millimeter, giving a more accurate index of red blood cell production than the reticulocyte percentage. Smears were also examined for the presence of nucleated red blood cells and for changes in the size of red blood cells. Hemoglobin was estimated by the Newcomer method and was recorded in percentage. On the glass standard used, as checked by blood iron determinations, 100 per cent was equivalent to 15.6 gm. of hemoglobin per 100 cc. of whole blood. Occasional white blood cell counts were done to detect any gross abnormalities.

Blood volume was calculated on all animals subjected to hemorrhage, using the values given for rabbits by Went and Drinker (9). Hemorrhage was produced by incising one of the large veins at the base of the ear, the blood being received in a graduated cylinder to determine the exact amount lost by the animal. The incisions healed quickly, and the same vein could be used repeatedly for successive bleedings. Blood destruction was induced in some of the animals by the use of acetyl phenylhydrazine. The drug was used in a solution containing 15 mg. per cc., and was injected subcutaneously.

The animals were quickly killed by a sharp blow on the back of the neck. One femur was immediately removed and split open. A small cross-section of marrow 2 or 3 mm. long was taken from the upper end of the shaft of the femur. This piece of marrow was at once emulsified in 2 per cent sodium citrate and smears were made according to the method described by Doan and Zervas (10). These cover-slip preparations were stained with brilliant cresyl blue at the time of smearing and were usually counterstained with Wright's stain. Some of the smears were counterstained with Jenner-Giemsa stain. Differential counts of 2,000 to 4,000 cells of the erythroid series were made on the smears of each marrow. The cells were divided into four groups: (a) mature erythrocytes, (b) reticulocytes, (c) normoblasts, and (d) erythroblasts and megaloblasts. The classification followed is that of Sabin (11).

The entire marrow of both femora and both tibiae was examined routinely. Longitudinal and cross-sections of femoral and, occasionally, of tibial marrow were fixed in Helly's fluid, sectioned, and stained with hematoxylin-eosin and Giemsa stains. Small portions of liver and spleen were also fixed, sectioned, and stained in the same manner.

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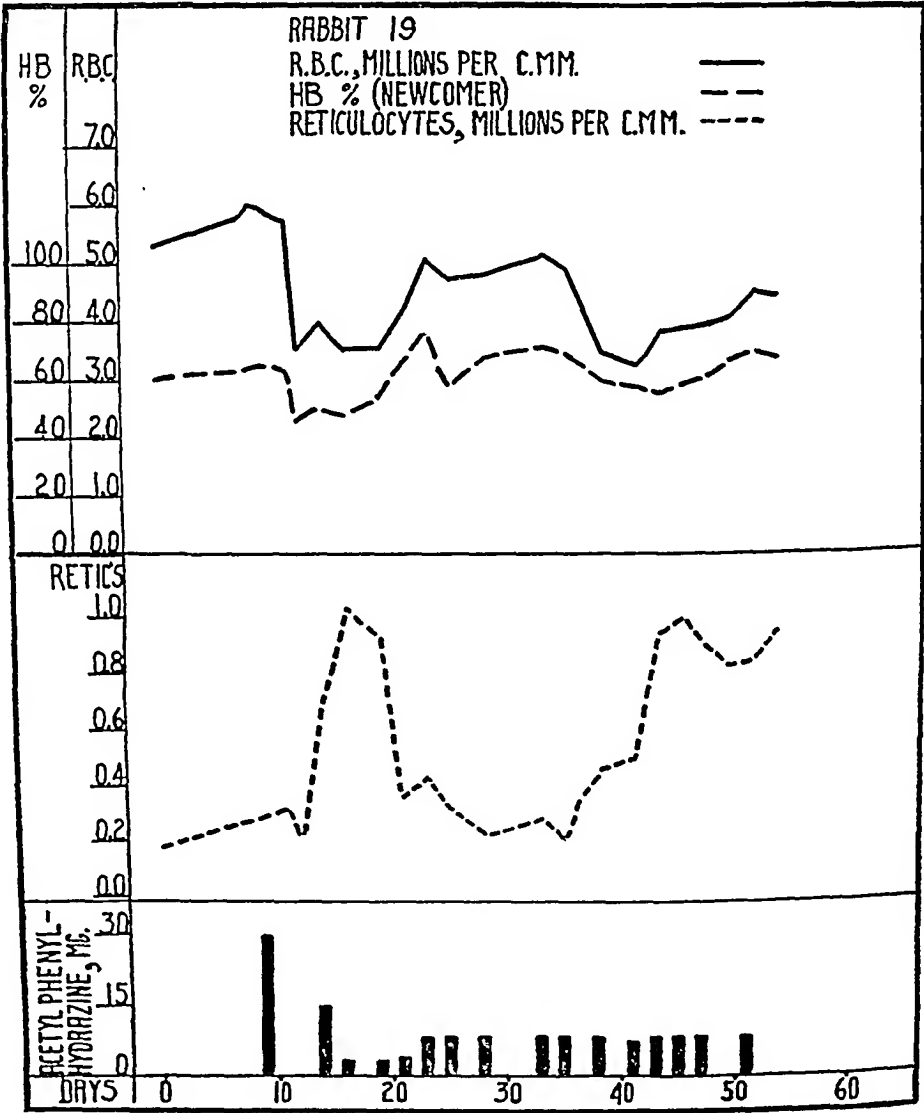


CHART 3. Observations on the blood of Rabbit 19 injected with acetyl phenylhydrazine.

the red blood cells showed a high degree of anisocytosis. Normoblasts were seen in a majority of the peripheral blood smears, but always in small numbers.

Late erythroblasts comprised much the greatest proportion of the erythroblast-megaloblast group; megaloblasts were extremely scarce.

In the gross, the marrow of these animals was firm and red, with small flecks of yellow fat throughout the upper three-fourths of the femur and upper third of the tibia. The lower fourth of the femur and lower half of the tibia were filled with yellow fatty marrow. Some

TABLE II

Peripheral Blood Values, Bleeding Data, and Marrow Findings in Five Rabbits Subjected to Acute Hemorrhage

Rabbit No.	Average of peripheral blood values before bleeding			Bleeding data		Lowest peripheral blood values reached after bleeding			Reticulocyte peak (No. per c.mm.)	Peripheral blood values immediately before death			Erythroid cells in bone marrow			
	R.B.C. per c.mm.		Absolute No. reticulocytes per c.mm.	Total cc. of blood removed	Total per cent of blood volume removed	R.B.C. per c.mm.		Absolute No. reticulocytes per c.mm.		R.B.C. per c.mm.		Absolute No. reticulocytes per c.mm.	Erythroblasts and megakoblasts	Normoblasts	Reticulocytes	Mature erythrocytes
	mil-lions	per cent				mil-lions	per cent			mil-lions	per cent					
8	6.20	72	0.13	100	50	1.91	30	0.31	1.10	2.90	45	0.86	8.5	49.0	30.5	12.0
11	5.54	75	0.09	55	26	2.80	33	0.07	0.70	3.51	46	0.68	8.1	42.8	33.6	15.5
12	4.88	72	0.13	93	35	3.02	39	0.12	0.71	3.93	47	0.71	8.5	31.8	27.2	32.5
14	6.28	74	0.21	70	41	2.87	38	0.21	0.80	4.11	51	0.76	5.1	46.4	31.6	17.0
18	5.86	76	0.18	105	39	2.01	35	0.12	1.02	3.66	59	1.02	9.0	49.1	28.0	13.9
Average.	5.75	74	0.15	85	38	2.52	35	0.21	0.87	3.62	50	0.81	7.8	43.8	30.2	18.2

slight individual variations in the extent of the red marrow were noted. Sections showed numerous large fat cells, even in the red marrow at the upper end of the femur, the marrow cells forming a network of thin strands and small clumps. Erythropoietic islands were seen but indistinctly.

The Findings in Acute Hemorrhage

After a period of several days for determination of normal blood values, the five animals included in this group were subjected to acute hemorrhage. In a single bleeding, or in two bleedings less than 12

animals before blood production was accelerated. Heath and Daland (13) have shown that reticulocytes probably mature to adult erythrocytes in from 4 to 6 days. In normal animals there is no urgent need for the liberation of immature cells into the circulation, and the maturation period of the reticulocytes is passed in part before leaving the marrow. Thus, in the marrow of normal animals, the reticulocytes tend to accumulate, and they greatly outnumber the normoblasts from which they are derived. On the other hand, animals that have suffered blood loss or blood destruction need every available red blood cell; reticulocytes are poured into the circulation soon after they are formed, and even an occasional normoblast escapes. In the marrow of these animals the reticulocytes, although present in a percentage approximating the normal, are much less numerous than their immediate precursors, the normoblasts. Therefore, when blood production is accelerated and the reticulocyte-normoblast ratio in the marrow is reversed, it seems probable that the reticulocytes pass the greater portion of their maturation period in the peripheral circulation. The high reticulocyte counts are, to some extent, due to this fact and are not entirely the result of increased cell production.

It must be borne in mind that certain errors are involved in the making of differential counts of the erythroid cells in the marrow. First, counts of cells in one standard portion of the marrow may not be representative of the state of the marrow as a whole. Second, in a series of cells which are rapidly maturing and constantly changing it is almost impossible to make wholly accurate classifications. This is especially true in the case of marrows in which erythropoiesis has been greatly accelerated and to a certain extent disorganized. Hence, differential counts of the erythroid cells in the bone marrow must be interpreted as indicative of general trends rather than as representative of absolutely accurate levels of red blood cell production.

The above experiments revealed four types of general trends in erythropoiesis. These types are graphically shown in Chart 4. The marrows of normal rabbits maintaining an average reticulocyte count of 0.12 million per c.mm. in the peripheral blood were characterized by the predominance of mature cells. The percentages of erythroid cells were, in fact, rather proportional to the degree of maturity of the cells. In all animals subjected to blood loss and blood destruction

of the blood volume was removed by bleeding, the average lowering of the red blood cell count was 56 per cent. This discrepancy may have been due to overdilution or redistribution phenomena, or it may indicate that the total blood volume figures are too high. The reticulocyte response in the peripheral circulation, as determined by the increase in the percentage, began within a few hours after bleeding. Owing to the progressive lowering of the red blood cell count, the absolute number of reticulocytes per cubic millimeter did not show an increase until the 2nd or 3rd day after bleeding. Early in the response numerous abnormally large reticulocytes, heavily laden with hemoglobin, appeared in the circulating blood; later there were small as well as large reticulated cells. This gave rise to a high degree of anisocytosis. Nucleated red cells appeared sporadically when the reticulocyte counts were high, and they were never present in numbers large enough to include in a percentage count. The peak of the response, as closely as it could be determined, was reached on the 4th to the 6th day after bleeding and varied from 0.7 to 1.1 million reticulocytes per c.mm. At this peak the average number of reticulocytes per c.mm. was 0.87 million, or 5.8 times the prehemorrhage level of 0.15 million. At the time of death the level was slightly lower, being 5.4 times the normal.

The counts of erythroid cells in the femoral marrow of these animals (Table II) revealed a reversal of the normal proportions existing between mature cells, reticulocytes, and normoblasts. Normoblasts were the predominant type of cell, many of them large and heavily laden with hemoglobin. The percentage of erythroblasts and megakaryoblasts was not changed appreciably from the normal.

In the gross, the marrow of these animals was softer, redder, and more friable than that of normal animals. Little or no extension of red marrow into areas normally occupied by yellow marrow was noted, except in Rabbit 18 in which the red marrow filled almost the entire cavity of the femur. The small flecks of yellow fat seen in the red marrow of normal animals were less numerous or absent. Microscopically, sections of these marrows showed a perceptible decrease in the number and size of the fat cells. The erythropoietic islands were definitely enlarged.

which maintained an average reticulocyte count 3.3 times the normal for a period of 40 days, the marrows were characterized by diminished percentage of mature erythrocytes, predominance of normoblasts, and nearly triple the normal percentage of erythroblasts and megaloblasts. The greater percentage of retaining the iron from the red blood cells destroyed within the body, in contrast to its loss in the chronic hemorrhage produced by the periodic removal of blood.

SUMMARY

The relative proportion of the various sorts of erythroid cells of the bone marrow has been determined after acute and chronic hemorrhage and after damage to the marrow with acetyl phenylhydrazine. The normal erythroid pattern shows megaloblasts and erythroblasts in the lowest percentage, then normoblasts, reticulocytes, and mature erythrocytes respectively, in increasing proportions. All three states studied show an increasing "shift to the left" up to a condition after acetyl phenylhydrazine, in which the erythroblasts and megaloblasts exceed the mature erythrocytes. The marrow pattern finds direct expression in terms of the cells of the blood.

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hemorrhage average of 0.13 million per c.mm. Immediately before death the average reticulocyte count was 0.68 million, or 4.8 times as high as the prehemorrhage level. At the termination of the period of bleeding, the average red blood cell count was 63 per cent and the average hemoglobin 69 per cent of their original values. Thus it seemed that, despite the average loss of 58 gm. of hemoglobin during

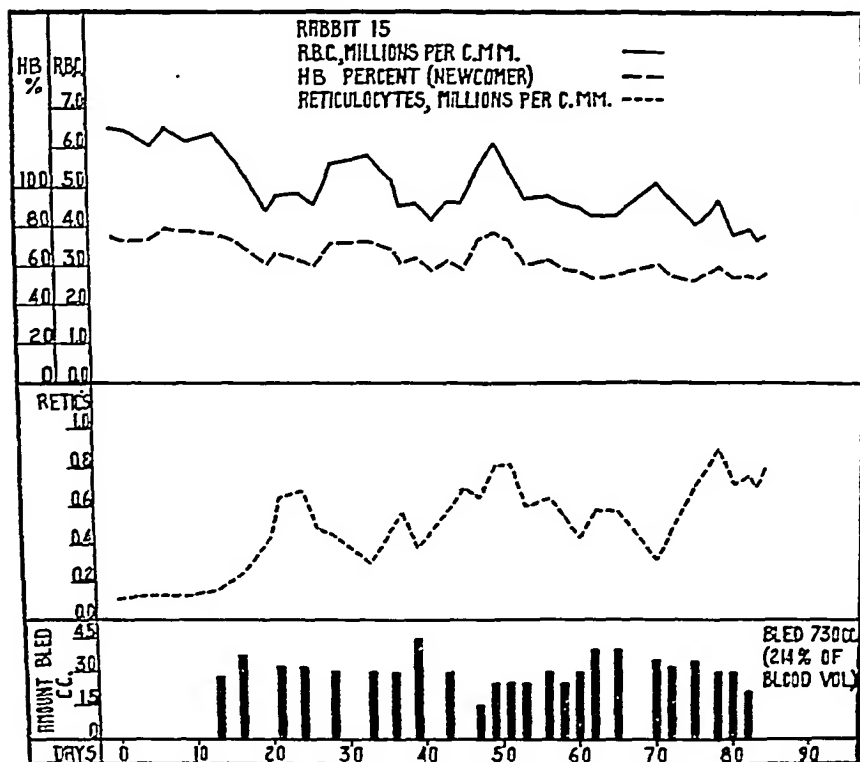


CHART 2. Observations on the blood of Rabbit 15 subjected to chronic hemorrhage.

the bleeding period, these animals were still able to produce red blood cells with a normal or slightly greater than normal quota of hemoglobin. Considerable anisocytosis was noted during the latter weeks. Nucleated red cells, always normoblasts, were frequently seen in the blood smears when the reticulocyte counts were high, yet they were never present in sufficient numbers to count.

discovery was extended at once to the treatment of hypophysectomized animals with these mixtures, the findings with normal animals being confirmed there (5).

The phenomenon of synergism has been confirmed in the recent publication of Leonard (6), who by employing hypophyseal extracts differently prepared has raised the question of the hypophyseal component responsible for the reaction. Leonard succeeded in obtaining excellent activation reactions by the employment of an extract low or indeed lacking in the growth hormone but notably rich in the gonad-stimulating substance.

The excellent synergism which was secured in 1931 resulted, as has been said, from the combination of prolan with growth-promoting hypophyseal extracts in which the gonad-stimulating hormone was so low as to be undemonstrable by the employment of quantities several times those used for the activation experiments. At the time of the first communication on this subject, gonad-stimulating extracts were made and tested for synergism with prolan. These extracts were, however, purposely diluted so that their gonad-stimulating effect would not preclude the recognition of the activation phenomenon. The diluted extracts of the gonad-stimulating hormone did not show the phenomenon, but it must be borne in mind that a concurrent dilution of the substance responsible for the reaction was thereby inevitably effected.

Continued study of the new phenomenon has been made. Results resembling those of Leonard have repeatedly been secured. In fact, hypophyseal extracts rich in the gonad-stimulating hormone can be secured by a variety of methods which show a well marked activation reaction with prolan. However, the reacting component of the anterior hypophysis is neither the gonad-stimulating nor the growth hormone. The present paper is concerned with the recognition and preliminary characterization of the hypophyseal substance involved in this reaction.

Non-Identity of the Hypophyseal Component with the Growth Hormone

Acetone-ammonia³ (5), aqueous pyridine, alcohol-ammonia (5) or simple alcoholic extracts of anterior lobe tissue can be produced which are

³ Many of the procedures which we have employed in this study were devised in the preceding work of Evans, Meyer and Simpson, and described in detail

first dose was smaller did not show as high an initial reticulocyte rise nor as low a subsequent drop.

During the period of administration of acetyl phenylhydrazine the average reticulocyte count was 0.53 million per c.mm., a value 3.3 times as high as the average count in the control period. At the time of death the average reticulocyte count was 0.81 million, or 5 times the control average. At the end of the injection period, the average

TABLE IV

Peripheral Blood Values, Injection Data, and Marrow Findings in Four Rabbits Injected with Acetyl Phenylhydrazine

Rabbit No.	Average of peripheral blood values before injection			Acetyl phenylhydrazine injections			Average of peripheral blood values during period of injection			Peripheral blood values immediately before death			Erythroid cells in bone marrow				
	R.B.C. per c.mm.	Hb	Absolute No. reticulo-cytes per c.mm.	No. of injections	Duration of period of injections	Total amount injected	R.B.C. per c.mm.	Hb	Absolute No. reticulo-cytes per c.mm.	R.B.C. per c.mm.	Hb	Absolute No. reticulo-cytes per c.mm.	Erythroblasts and megakoblasts	Normoblasts	Reticulocytes	Mature erythrocytes	
	mil-lions	per cent	mil-lions		days	mg.	mil-lions	per cent	mil-lions	mil-lions	per cent	mil-lions	per cent	per cent	per cent	per cent	
19	5.78	63	0.25	16	41	163	4.24	60	0.59	4.45	68	0.94	17.1	32.3	28.8	21.8	
20	5.29	56	0.16	16	41	152	4.09	52	0.53	3.86	51	0.63	17.6	33.7	29.6	19.1	
21	5.14	72	0.11	18	40	201	4.26	63	0.47	3.18	53	0.80	20.6	51.9	21.6	5.9	
23	5.48	64	0.13	18	40	172	4.15	55	0.53	3.51	53	0.86	17.6	46.5	26.5	9.4	
Average.	5.42	64	0.16	17	41	172	4.19	58	0.53	3.75	56	0.81	18.2	41.1	26.6	14.1	

red blood cell count was only 69 per cent while the average hemoglobin was 87 per cent of the respective control period values. This relative increase of hemoglobin in relation to red blood cell count was paralleled by an increase in the average diameter of the red blood cells. There was also an increase in the hemoglobin content of many of the red cells, judging from their appearance in stained smears. The higher hemoglobin percentage might be accounted for by the fact that in the acute and chronic hemorrhage experiments there was a loss of iron, while, in the anemia produced by the administration of phenyl-

Growth-promoting extracts from the hypophysis can be produced devoid of the factor concerned in synergism. In the previous paper (4, Table 8) two types of purified growth hormone preparations made by acetic acid extraction and by repeated isoelectric precipitation were cited as potent in the reacting substance. Preparations of the growth hormone made in this way have, as a matter of fact, usually given excellent synergism when combined with prolan but we now know that this is not invariably the case; though potent growth-stimulators, they may fail to give the activation phenomenon. Finally, flavianic acid precipitates (5) from acidified alkaline extracts of beef anterior lobe, when extracted with alcohol-ammonia to remove flavianic acid, furnish growth hormone preparations devoid of the synergistic factor.

Non-Identity of the Hypophyseal Component with the Gonad-Stimulating Hormone

When the synergistic factor and the gonadotropic hormone are present together, three important types of results are obtained, depending on the relative concentration of the two components in the fraction in question. Further, it can be shown that dilution of various hypophyseal extracts does not decrease the content of the two components in a parallel manner.

When dealing with high doses of concentrates potent in the synergistic factor, but contaminated with large amounts of the gonad-stimulating hormone, the activation phenomenon cannot be recognized, for the test animal is already near the limit of its reactivity to these substances. Activation is, however, readily demonstrated at the lower dose levels. Thus synergistic effects can only be shown when ovary weights (calculated by summation of the prolan and hypophyseal gonadotropic hormone effect) do not exceed a certain maximum of 60-80 mg. and it is therefore best to use a dose of prolan which will give 30-40 mg. ovaries. The fact that high dosage of gonadotropic hormone obscures the activation phenomenon accounts for the apparent anomaly of increased percentage activation with decrease in dose, shown in Table II.

Preparations containing large quantities of gonad-stimulating hormone and relatively small amounts of the synergic substance are occa-

(which extracts very little growth hormone) followed by treatment with formic acid (which destroys the gonad-stimulating hormone) yields a water-soluble preparation, rich in the synergistic factor and free of growth-promoting and gonad-stimulating hormones. Examples of such preparations are included in Tables I and III.

TABLE III

Effect of the Combination of Prolan with Hypophyseal Preparations Containing Low Amounts of Gonadotropic Hormone

Hypophyseal component			Prolan		Combination	
Preparation	Dose	Weight of ovaries	Dose	Weight of ovaries	Weight of ovaries	Activation
	mg.	mg.	mg.	mg.	mg.	per cent
Acetone-ammonia powder (beef) R1346	54.4	20	27.3	28	108	260
	27.3	18			47	68
	13.6	13			37	0
Alcohol-ammonia powder treated with formic acid; water-soluble fraction (sheep) R1475	27.3	25	27.3	41	85	77
	13.6	29			91	75
	6.8	24			100	114
	2.7	19			107	155
	1.4	14			80	116
Acetic acid powder (sheep) R1076	54.4	16	27.3	24	50	127
	27.3	17			30	30
	13.6	16			28	27
Acetic acid powder water-soluble fraction (sheep) R1193	13.6	19	27.3	32	63	91
	6.8	13			52	92
	1.4	18			33	0
Acetone-ammonia water-soluble fraction (sheep) R1217	13.6	18	27.3	28	84	200
	6.8	15			67	168
	1.4	14			29	21

Characteristics of the Hypophyseal Substance

Since the active principle present in the fractions so far obtained has been inevitably associated with protein, it has been impossible to draw any conclusions regarding its chemical reactions or physico-chemical properties. However, as found in the protein fractions it was soluble in water and was precipitable by flavianic, phosphotungstic

The differential counts of erythroid cells in the marrows of these animals again revealed the normoblast as the predominant type of cell. The percentage of reticulocytes was approximately normal; the mature erythrocytes were much reduced. The most striking fact shown by the differential counts was the great increase of the erythroblast-megaloblast group to 18.2 per cent, nearly 3 times the normal value for these cells. This increase was chiefly due to large numbers of erythroblasts, although megaloblasts were also frequently seen. Some disorganization of the erythropoietic process was suggested by the great variation in size of the erythroblasts and normoblasts.

In the gross, the red marrows of these animals appeared to be extremely hyperplastic, but did not show much more extension than did the marrows of chronic hemorrhage animals. Red marrow filled the entire cavity of the femur and the proximal half of the tibia, and in two of the animals small streaks of red marrow extended down through the fatty marrow in the distal half of the tibia. The marrow was rich red in color, somewhat darker than normal, and was exceedingly soft, being almost liquid in the upper third of the femur. No flecks of gross fat were visible in the femoral marrow.

In sections the fat cells were seen to be greatly diminished in size and number and in many areas they were entirely absent. Large confluent groups of erythrocytic cells were present in great numbers throughout the sections. Centers of intense myeloid activity were also evident.

The spleens of these animals were from 2 to 3 times the normal size and of a chocolate-brown color. The organs cut with much decreased resistance. An abnormally soft, mushy parenchyma bulged from the cut surface and no typical splenic structure could be seen. Sections of all four spleens showed distinct areas of erythropoietic activity. Although the livers were normal in the gross, microscopic sections showed a few small areas of toxic degeneration and leucocytic infiltration.

COMMENT

An interesting question of accuracy arises when an attempt is made to compare the high reticulocyte counts in the blood of animals having increased blood production with the reticulocyte counts in the same

prolan ovary, characterized by a small crop of corporea lutea, was in marked contrast to the type of ovary found after injection of prolan in combination with the synergistic factor. In the latter case, the ovary contained large numbers of corporea and large follicles, so many that frequently they were not easily counted under the binocular.

Preparation of Powders Containing the Synergistic Substance

Desiccated Glands.—Carefully dissected fresh anterior lobes were placed under dry acetone for 1 week with daily changes of acetone. They were then ground, rapidly dried in a heated vacuum desiccator and then dried over phosphorus pentoxide *in vacuo*.

Acetone Powders.—Standard alkaline extract (5) was poured into ten volumes of cold acetone and the precipitate isolated by centrifuging and drying as above.

Alcohol-Ammonia.—Powders containing a high concentration of the active substance have been prepared by the use of 50 per cent alcohol containing 1 per cent ammonia. 20 gm. of sheep acetone powder was extracted for 18 hours at room temperature with 1000 cc. of 50 per cent alcohol containing 1 per cent ammonia. The solution was centrifuged and the residue reextracted with 800 cc. of alcohol-ammonia for 8 hours. The solution was again centrifuged and the combined supernatants poured into 6000 cc. of alcohol. Acetic acid (3–5 cc.) was added to aid precipitation. The product was isolated as previously described; yield, 5.7 gm.

From 30 gm. of beef acetone powder there was obtained, similarly, 10 gm. of the alcohol-ammonia powder.

Pyridine Extracts.—In this method, 50 per cent aqueous pyridine was used as described for alcohol-ammonia powders. A similar procedure was described by Fevold, Hisaw and Leonard (7). As suggested by these authors, the pyridine powders were extracted with water and the active substance was found concentrated in the water-soluble fraction. The yield of water-soluble material from 20 gm. of desiccated sheep glands was 0.3 gm. and was highly potent. 10 gm. of sheep or beef acetone powder gave 1.0 and 1.4 gm. respectively of the water-soluble material and was somewhat less potent than the concentrates obtained from desiccated sheep glands.

Aqueous Alcohol.—200 gm. of frozen fresh beef anterior lobes was extracted with 1000 cc. of 40 per cent alcohol for 4 hours and the insoluble material was reextracted with 500 cc. of 40 per cent alcohol for 18 hours. The mixture was centrifuged and the supernatant poured into 5000 cc. of alcohol. Saturated alcoholic sodium acetate was added to aid precipitation and after centrifuging and drying, there was obtained 2.3 gm. of highly potent concentrate.

Trichloroacetic Acid.—Frozen fresh beef anterior lobes (190 gm.) were extracted for 4½ hours with 600 cc. of 1 per cent trichloroacetic acid. The insoluble material was reextracted for 18 hours with 500 cc. of 1 per cent trichloroacetic acid and the

there were definite changes in the marrow pattern. In animals studied during the recovery from acute hemorrhage, when the average reticulocyte count was at a peak 5.4 times the control level, the marrows showed a great diminution of mature erythrocytes, an increase of normoblasts to predominance, and no appreciable change in the percentage of erythroblasts and megablasts.

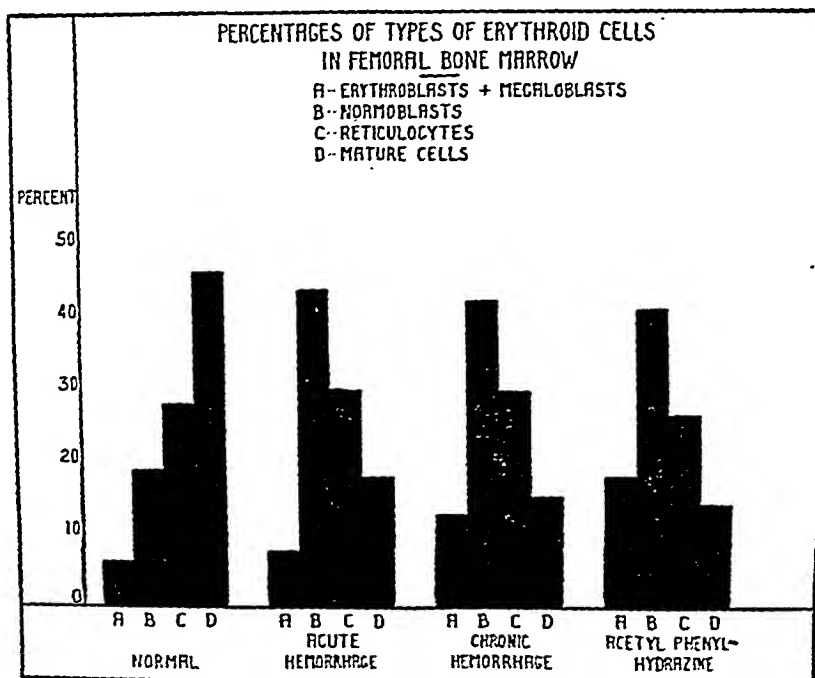


CHART 4. Percentage of types of erythroid cells observed in the femoral bone marrow of normal rabbits, of those subjected to acute and chronic hemorrhage, and of those in which acetyl phenylhydrazine was employed.

In animals which were subjected to chronic hemorrhage and which maintained an average reticulocyte count 4 times the normal for an average period of 72 days, the marrows were characterized by diminished percentage of mature erythrocytes, predominance of normoblasts, and double the normal percentage of erythroblasts and megablasts.

In animals which were injected with acetyl phenylhydrazine and

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THE HYPOPHYSEAL SUBSTANCE GIVING INCREASED GONADOTROPIC EFFECTS WHEN COMBINED WITH PROLAN*

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In 1931 (1, 2), publication was first made from our laboratory of a prolonged series of experiments which disclosed a singular ineffectiveness of prolan¹ when administered to hypophysectomized animals.² In seeking an explanation of these facts it seemed likely that the effect of prolan on normal young animals must therefore be strengthened by some element furnished by the animal's own hypophysis. An endeavor was hence made to increase the prolan effect in normal young animals by combining prolan with hypophyseal extracts. A clear-cut synergism was demonstrated by combining the two substances (3, 4). The first results were secured by combining with hypophyseal extracts containing the growth hormone, and from preliminary considerations it seemed probable that the growth hormone in the growth-promoting extracts was responsible for the new phenomenon. The

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¹ For the sake of convenience, we have employed this term (after Zondek) to designate the substance in the urine of pregnant women which precociously stimulates ovarian development in immature rats or mice.

² So impressive indeed was this experience that it at first indicated the total capacity of prolan, regardless of dose, to effect the sexual system of hypophysectomized animals, although later work showed that this result could be obtained with extremely high dosage (2, 5).

rabbit used. Nichols (2) stated that there appears to be choice in rabbits in regard to color, the general adaptability being white, gray, brown, and black. Finkelstein (3) found the Himalayan rabbit more susceptible to initial infection than other groups although he made no statement as to whether these latter represented mongrels or definite breeds. Working with a family of silver rabbits, Frei (4) noted that they developed a significantly higher generalized lesion rate than other types of rabbits, and concluded that they were constitutionally susceptible. More recently Frazier and Mu (5) came to a similar conclusion regarding the albino rabbit, after observing that this variety offered less resistance to syphilitic infection than did a brown variety.

In general, reference to variations in susceptibility, attributed to the kind of rabbit used, have been based on color varieties rather than on standard breeds or on results obtained from some particular breed or family. So far as is known, no effort has been made to compare the reaction to infection of representative standard breeds or families of rabbits in a systematic manner. The experiments reported in this paper differ from those reported by others in that the animals used were from carefully selected standard breeds of rabbits which have been propagated in pure line in our own laboratories; complete records are available for all animals used, and the comparisons made are based on all phases of the reaction to infection from incubation to the onset of spontaneous recovery. Moreover, especial attention has been given to generalized manifestations of the disease, including the kind, number, destructiveness, and persistence of lesions.

Material and Methods

Comparisons of the reaction to syphilitic infection, as measured by the course and severity of disease, have been made on pure bred rabbits of ten standard breeds. These include Havana, Dutch, English, Himalayan, Rex, Belgian, Polish, Blue Beveren, and New Zealand Red. The results to be reported are limited to two experiments, both of which contained animals of the first five breeds enumerated, giving a total of 57 rabbits.

All of the animals were raised in this laboratory with the exception of three Rexes used in the second experiment, and these were bred by a laboratory technician from laboratory stock. The animals were carefully selected healthy males averaging 6 months of age. They were kept in individual cages in a well ventilated room receiving diffuse north light, and were fed a uniform diet consisting of hay, oats, and a commercial food pellet with a free supply of water. The animals of the first experiment were inoculated on Oct. 26, 1931, and those of the second on Mar. 31, 1932. Both series were observed over a period of 3

extremely low in or devoid of growth hormone though high in gonad-stimulating properties. Such extracts show excellent synergism when combined with prolan. The results obtained by the combination of prolan with this type of hypophyseal preparation are summarized in Table I.

TABLE I

Effect of the Combination of Prolan with Hypophyseal Preparations Containing Little or No Growth Hormone

Hypophyseal component			Prolan		Combination	
Preparation	Dose*	Weight of ovaries	Dose	Weight of ovaries	Weight of ovaries	Activation
	mg.	mg.	mg.	mg.	mg.	per cent
Acetone-ammonia, water-soluble fraction (sheep) R1217	13.6	18	27.3	28	84	200†
Aqueous pyridine extract of acetic acid powder, water-soluble fraction (sheep) R1181	13.6	12	27.3	26	43	115
Aqueous pyridine extract of acetic acid powder, water-soluble fraction (sheep) R1072	7.6	18	27.3	24	80	230
Alcohol-ammonia (sheep) R1305	2.7	38	27.3	40	108	80
Aqueous alcohol (beef) R1456	6.8	15	27.3	31	87	212
Trichloroacetic acid, water-soluble fraction (beef) R1463	6.8	20	27.3	31	81	146

* Total dose given to each animal in 3 days.

† Activation is arbitrarily calculated as follows:

$$\frac{C - (P + H - I)}{P + H - I} \times 100 = \text{per cent activation; e.g.,}$$

$$\frac{84 - (28 + 18 - 18)}{28 + 18 - 18} \times 100 = 200 \text{ per cent.}$$

C = average weight of ovaries of the group receiving the combination of prolan and hypophyseal component.

P = average weight of ovaries of the group receiving prolan alone.

H = average weight of ovaries of the group receiving hypophyseal preparation alone.

I = average weight of infantile ovaries of uninjected controls. In all calculations in this paper the value of (I) is taken as 18 mg.

in their monograph (5), publication of which has unfortunately been somewhat delayed. In all instances of the utilization or modification of these preceding methods, acknowledgment is made by specific reference to the monograph.

age deviations in the two experiments gave the values on which Text-fig. 1 was constructed. Text-fig. 2, constructed on the same basis, represents the mean time of occurrence of the different disease phenomena in each breed group, expressed as combined percentage deviations from the mean values for all the animals of each experiment as

TABLE I
Incidence of Various Phenomena of Infection and Focal Distribution of Generalized Lesions

Experiment No.	Breed	No. of animals	Primary orchitis	Critical edema	Metastatic orchitis	Distribution of generalized lesions		
						Incidence	Focal distribution	
							Actual	Relative
1	Havana	4	per cent	per cent	per cent	per cent		
	Dutch	5	100	100.0	75.0	75.0		
	English	5	100	60.0	60.0	100.0	5.3	4.0
	Himalayan	9	100	100.0	100.0	100.0	4.4	4.4
	Rex	3	100	88.8	100.0	88.8	6.2	6.2
			100	100.0	100.0	100.0	11.3	10.0
Total...		26	100	88.5	88.5	92.3	16.7	16.7
2	Havana	6	100	50.0	50.0	0	8.7	8.0
	Dutch	6	100	50.0	66.7	0		
	English	6	100	66.7	83.3	16.7	0	0
	Himalayan	6*	100	50.0	80.0	50.0	1.0	0.2
	Rex	7	100	28.6	71.4	83.3	2.7	1.3
						57.1	3.2	2.7
Total...		31	100	48.4	70.0	41.9	1.3	0.7
							2.3	1.0

*One animal was a unilateral cryptorchid.

shown in Table II. Text-fig. 3 represents graphically values which are given in Tables III, IV, and VI.

DISCUSSION

Before discussing the results of these experiments, attention should be called to the difference in severity of the disease in the two series. In general, it was severe in the first and very mild in the second. This difference may readily be seen by comparing the total mean values for

sionally encountered and when this condition obtains, the slight synergism shown at higher levels disappears entirely with lower dosage (Table II).

When powders containing large amounts of the active principle and very low amounts of the gonad-stimulating hormone (beef or chemically treated sheep preparations) are combined with prolan, the activation is roughly proportional to the dose of the reacting hypophyseal

TABLE II

Effect of Combination of Prolan with Hypophyseal Preparations Containing a Large Amount of Gonadotropic Hormone

Hypophyseal component			Prolan		Combination	
Preparation	Dose	Weight of ovaries	Dose	Weight of ovaries	Weight of ovaries	Activation
	mg.	mg.	mg.	mg.	mg.	per cent
Acetone powder (sheep) R1288	54.5	48	27.3	38	65	0
	27.3	34			62	15
	13.6	24			72	63
	6.8	19			75	92
Flavianate from isoelectric supernatant (sheep) R1173	54.5	78	27.3	32	107	16
	27.3	44			63	9
	13.6	27			73	78
	6.8	17			64	106
Flavianate from isoelectric supernatant (sheep) R986	54.5	72	27.3	53	111	4
	27.3	54			112	26
	13.6	38			89	22
	6.8	29			80	25
	2.7	18			52	0

substance (largely independent of the amount of prolان). This is illustrated in Table III.

The hypophyseal substance can be produced relatively free of both growth and gonad-stimulating hormones. By combination of the essential features in the procedures for preparation of the synergic substance free of the growth and gonad-stimulating hormones, the substance can be prepared free of both contaminants. For example, extraction of crude pituitary preparations with alcohol-ammonia

ment is not definitely known. The period of the year is, however, suggestive since during the late spring and early summer months when this group was observed, the disease is not infrequently less severe than during the autumn and winter. These differences, however, do not affect breed comparisons since all breeds were represented in both experiments.

The observations on the different breeds will now be considered from the standpoint of the sequence of events of the disease. For this purpose the mean values obtained by combining the two series and considering them as one experiment will be analyzed since a detailed dis-

TABLE III
Incidence and Mean Time of Occurrence of Primary Orchitis, Critical Edema, and Metastatic Orchitis in Combined Experiments

Difference of Primary Orchitis, Critical Edema, and Metastatic Orchitis in Combined Experiments								
Breed	Primary orchitis		Critical edema			Metastatic orchitis		
	Incidence	Incubation period	Incidence	Time of occurrence		Incidence	Time of occurrence	
				From inoculation	From primary orchitis		From inoculation	From primary orchitis
	<i>per cent</i>	<i>days</i>	<i>per cent</i>	<i>days</i>	<i>days</i>	<i>per cent</i>	<i>days</i>	<i>days</i>
Havana.....	100.0	21.0	70.0	34.2	13.0	60.0	65.5	44.3
Dutch.....	100.0	20.3	54.5	25.3	5.7	63.6	57.3	37.9
English.....	100.0	22.0	81.8	30.2	9.0	90.9	49.1	27.0
Himalayan.....	100.0	19.4	73.3	28.2	8.0	92.9	57.7	38.8
Rex.....	100.0	18.2	50.0	25.2	7.2	80.0	49.4	28.3
Total.....	100.0	20.0	66.7	28.9	8.7	78.6	55.2	32.5

cussion of the behavior of individual animals or of each experiment leads to the same conclusions. It should be pointed out that the manifestations of any particular phase of the disease are dependent on the degree of protection built up in the preceding reactive states.

Primary Orchitis.—(Table III.) A primary orchitis developed in all the animals. The mean incubation period for the combined experiment was 20 days. The variation of the values for the different breed groups from the combined mean was inconsiderable and probably not significant. However, in the Rex group the primary orchitis was

and sulfosalicylic acids. It was precipitable by the addition of alcohol to a concentration of 80 per cent in the presence of a slight salt concentration. The substance was surprisingly heat-stable when purified to this extent, although in solutions contaminated with growth hormone it was reported (3, 4) to be destroyed in 30 minutes at 70–80°C. It has now been found possible to heat the substance in neutral solution for 2 hours at 70°C. or to boil it for 30 minutes with only slight reduction in activity. The stability of the substance is also shown by the fact that all procedures for its preparation, some of them continuing for several days, were carried out at room temperature. Great stability toward atmospheric oxygen was also notable.

In order to obtain the principle free of gonad-stimulating hormone, use has been made of the destructive action of acetic and formic acids. In this respect, formic acid was found to be more advantageous, for the protein-like hypophyseal hormones were readily and completely soluble in the reagent. Two hours' contact with formic acid almost completely destroyed the gonadotropic hormone and definitely injured the growth hormone, but was practically without effect on the synergic substance. When acetic acid was used, longer treatment (6–18 hours) was necessary. With higher concentrations of acetic acid (96–98 per cent) the hormones were not completely soluble, while with lower concentrations (90 per cent) the gonad-stimulating hormone, although soluble, was not completely destroyed.

The activating principle was not ultrafilterable through membranes prepared from 6 per cent collodion supported on unglazed porcelain (neutral solution).

Biological Assay

The biological assay was conducted as follows:

A given number of milligrams of prolan, hypophyseal extract or the combination of the two was dissolved in 11 cc. of slightly alkaline water and adjusted to neutrality before injection. Each of three 22–26 day old rats received subcutaneously 1 cc. of the solution daily on 3 consecutive days. Autopsy was performed 96–100 hours after onset of injection and notes taken on ovaries and uterus and the ovaries were weighed. The average weight of two ovaries was taken as the degree of reaction of a given group. In some cases where activation was not certain from the ovary weights it was possible to decide that a significant activation had occurred by consideration of the type of ovarian reaction. The

metastatic orchitis were the highest of all the breeds. Thus it would seem that the reaction engendered by the primary orchitis and critical edema was insufficient to inhibit the development of metastatic orchitis in these two breeds.

The time of occurrence of the metastatic orchitis serves as another index of the effectiveness of the immunity produced by the previous course of the infection. With a comparable low incidence of edema in the Rex and Dutch groups (Table III), the Rex developed an orchitis of the uninoculated testicle at a mean time of 49.4 days after inoculation, considerably earlier than the Dutch group in which this lesion was retarded until the late mean time of 57.3 days. In the case of the Havana animals which showed a high incidence of edema and a low incidence of metastatic orchitis, the marked delay of the latter lesion, 65.5 days, is further evidence of the development of a highly efficient reaction. The mean time of metastatic orchitis in the English group was the earliest of any breed, 49.1 days; it will be recalled that the high edema rate was followed by a high incidence of uninoculated testicle involvement. The Himalayan animals, with a lower incidence of edema than the English, developed metastatic orchitis somewhat later. These several relationships are substantially the same when instead of calculating the time of appearance of metastatic orchitis from the date of inoculation, the interval between the primary and metastatic orchitis is determined.

Generalized Lesions.—(Table IV.) The presence or absence of generalized lesions is without doubt the most important single index of differences in the reaction to the disease. In the Havana and Dutch groups the incidence and relative focal distribution of generalized lesions were lower than in the others. This result is in keeping with the observations on the incidence and time of occurrence of the preceding phases of infection just discussed. The immunity developed by the Dutch and Havana animals persisted into the stage of generalized lesions, the incidence for example being 54.5 and 30.0 per cent. In the Himalayan, English, and Rex groups on the other hand, the defences built up in the previous reactive states were not of sufficient intensity or duration to offer an adequate protection against the development of generalized lesions. The incidence of secondary lesions in these groups was 72.7, 86.7, and 70.0 per cent respectively. Simi-

solutions were centrifuged. The combined supernatants were poured into 5500 cc. of alcohol and a few cc. of normal sodium hydroxide was added to facilitate precipitation. The product was isolated and dried as usual. The water-soluble fraction (0.8 gm.) from this concentrate was highly potent in the synergic principle.

Active Hypophyseal Substance Relatively Free of Growth and Gonadotropic Hormones.—The use of extractives as described above almost certainly eliminates the growth fraction (5, 6) and by the use of glacial formic or acetic acids it was possible to practically eliminate the gonadotropic factor. Crude alcohol-ammonia powder (6.3 gm.) was dissolved in 75 cc. of formic acid and allowed to stand 1 hour at room temperature and then 250 cc. of acetone was added slowly with vigorous stirring. The mixture was centrifuged and the precipitate was washed with acetone and dried; yield, 6.0 gm. This material was then extracted twice with 100 cc. portions of water (adjusted to neutrality), the insoluble material was centrifuged off and the supernatant precipitated by the addition of 1000 cc. of alcohol. Precipitation was aided by the addition of a small amount of a saturated alcoholic solution of sodium acetate. The material was isolated as before and the yield of water-soluble powder was 2.8 gm.

Acetic Acid Powder.—Growth-promoting preparations were often prepared (5) by precipitation of aqueous solutions of acetone powder at pH 5.2. When such fractions were further treated with glacial acetic acid for 6–18 hours, most of the gonadotropic hormone was destroyed with little harm to the synergic substance. The acetic acid powders thus obtained were then extracted with aqueous pyridine or aqueous ammonia and subsequently converted into water-soluble concentrates. The yields were small but the products were highly potent and relatively free of growth and gonadotropic hormones.

SUMMARY

The increased gonadotropic effect obtained by combining prolactin with hypophyseal extracts can be secured equally well by the use of preparations which contain high amounts of the growth or of the gonad-stimulating hormone.

In hypophyseal extracts containing the growth hormone, the synergistic effects do not parallel the content of growth-promoting substance.

Similarly, in hypophyseal extracts containing the gonad-stimulating hormone, the synergistic effects do not parallel the content in the gonadotropic factor.

The hypophyseal substance involved has been prepared sufficiently free of the growth and gonad-stimulating hormones to make it inadmissible to consider either of these substances as responsible for the reaction.

sponse with the development of lesions. This interval (Table IV) was approximately one-half as long in the Havana (12.7 days) and Dutch (14.8 days) groups as it was in the English (25.8 days), Himalayan (24.6) days, and Rex (23.6 days). These figures give an excellent idea of the efficiency of the reaction in the different breeds.

The character and location of generalized lesions in the several breeds may be used as a further basis for comparison of the differences in their reactions to the syphilitic infection (Table V). It will be seen that the proportion of bone to other lesions in the English and Rex groups was much higher than in any other breed. However, the type of bone lesion seen in these two groups was quite different. The Rex

TABLE V
Location of Generalized Lesions in Five Breeds of Rabbits—Combined Results

Breed	Total No.	Bone		Skin		Eye	
		No.	per cent	No.	per cent	No.	per cent
Havana.....	16	11	68.8	4	25.0	1	6.3
Dutch.....	23	14	60.9	9	39.1	0	—
English.....	36	32	88.9	3	8.3	1	2.8
Himalayan.....	106	50	47.2	54	50.9	2	1.9
Rex.....	55	42	76.4	13	23.6	0	—
Total.....	236	149	63.1	83	35.2	4	1.7

animals developed bone lesions that were destructive and invasive and were further characterized by very slow healing. In the English group, the lesions though persistent, were small and not destructive. The Himalayan group showed the lowest proportion of bone lesions and the Havana and Dutch rabbits occupied an intermediate position. In only one group, the Himalayan, was the majority of lesions located in the skin. These were the most malignant skin lesions seen; they were invasive, persistent, tended to ulcerate, and furthermore, many developed in unusual locations such as at the base of the ears and over either humerus.

With reference to the incidence and destructiveness of the bone lesions in Rex rabbits, it should be pointed out that the Rex rabbit occupies a unique position. It is regarded as a constitutionally de-

THE REACTION OF STANDARD BREEDS OF RABBITS TO EXPERIMENTAL SYPHILIS

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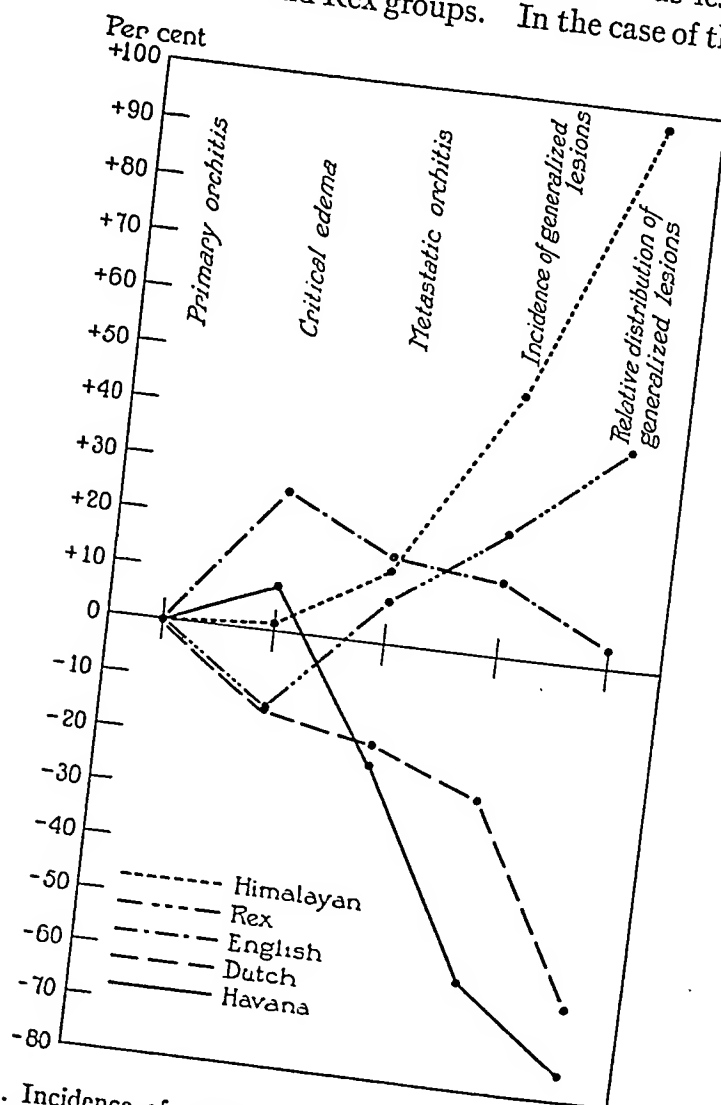
For several years a colony of standard bred rabbits has been maintained by this laboratory as the basis for the study of problems in constitution. The foundation stock for the colony was built up from pedigreed and registered animals purchased in this country and abroad. The breeds represented were chosen mainly on the basis of physical conformation, and the stock includes a full range of contrasting types from the small Polish to the Giant Flemish, and the cobby Dutch to the racy Belgian. Representative color varieties and coat characters are also included.¹

One of the purposes for which this colony was organized was to provide material of known quality for the study of relations between constitution and susceptibility to disease (1). As an approach to these problems, experiments were carried out with pure bred rabbits of various lines to determine, on the one hand, whether such differences as are represented by breed characters may be associated with distinctive differences in the reaction to disease-producing agents, and on the other, for the purpose of selecting the most suitable material for a more detailed study of any relations which might be found. The present report is concerned with observations on the response of different breeds of rabbits to one of the agents employed, namely, *Tr. pallidum*.

From time to time there has appeared in the literature of experimental syphilis suggestions that resistance to infection may be related to the breed or color of the

¹ For a description of American breeds and standards of perfection, the reader is referred to the Guide Book issued periodically by The American Rabbit and Cavy Breeders Association, Inc., 7408 Normal Avenue, Chicago, Ill. Breeds recognized in other countries vary somewhat from those in America, but the standards agree in most essential respects.

ing an efficient mechanism of protection against the clinical manifestations of syphilis. A similar state of protection was less successfully achieved in the English and Rex groups. In the case of the Himalayan



TEXT-FIG. 1. Incidence of various phenomena of infection and relative distribution of generalized lesions. Calculated as combined percentage deviation from mean values in two experiments.

animals, with the lowest incidence of healed lesions, the reaction to the infection was clearly the least efficient.

What has been said concerning the course of the infection in the

months when the main experiments were terminated. Selected groups were, however, kept under observation for 6 months. The Nichols' strain of *Tr. pallidum* was employed in both experiments. The inoculation was made into one testicle, using 0.3 cc. of a saline emulsion prepared from an actively developing testicular lesion and containing from two to four organisms per dark field. The different breeds were inoculated alternately, and no two animals of the same breed received material from the same syringe.

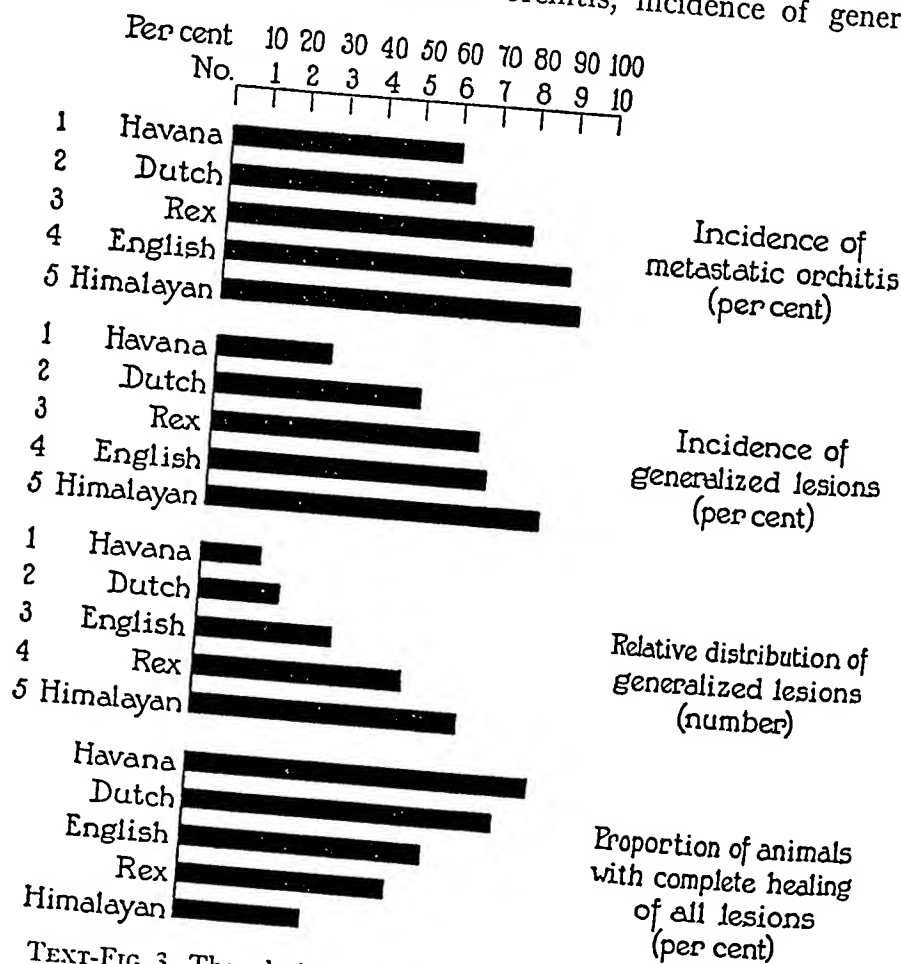
The evaluation of the character and significance of the reaction to infection was based upon a consideration of various clinical phenomena. Special attention was paid to the following: (1) the frequency and time of occurrence of the primary orchitis; (2) the frequency and time of occurrence of critical edema in the inoculated testicle; (3) the frequency and time of occurrence of lesions in the uninoculated testicle (metastatic orchitis); (4) the frequency, time of occurrence, and number of generalized lesions of the skin, bones and periosteum, and eyes; (5) the proportion of animals that showed complete healing of all lesions at the termination of the 3 months period of observation.

In the analysis of the results group means have been employed, a procedure which tends to minimize the effect of the chance occurrence of a case of severe syphilis in any one group. By the designation "focal distribution" of generalized lesions is meant the number of discrete foci at which lesions developed as determined by actual count. The figures for actual distribution represent the mean values for those animals of the group in which generalized lesions actually developed, while the values for relative distribution indicate the results in terms of the entire group.

RESULTS

The results are recorded in Tables I to VI and Text-figs. 1 to 3; in each instance the observations are presented from the standpoint of the standard breed of rabbit under consideration, namely, the Havana, Dutch, English, Himalayan, and Rex. Tables I and II give the incidence and mean time of occurrence of various phenomena of infection and the actual and relative focal distribution of generalized lesions of both experiments, together with the mean values for the total number of animals in each experiment. Tables III to VI contain values which have been obtained by combining the two experiments and treating them as one. Text-fig. 1 represents the incidence of the disease phenomena in terms of percentage deviation from the mean values of each experiment. In each experiment, the frequency of succeeding stages of infection in each breed group was calculated as percentage deviation from the mean values for all the animals of the experiment as given in Table I. The mean of the algebraic sum of the breed group percent-

As has been said, the values for the incidence and number of metastatic lesions furnish the best evidence of differences in the response to infection. In Text-fig. 3 a numerical value of 1, 2, 3, 4, or 5 was assigned to each breed group according to its position in an ascending scale of incidence of metastatic orchitis, incidence of generalized



TEXT-FIG. 3. The relation of rabbit breed to experimental syphilis.

lesions, and relative focal distribution of generalized lesions. The sum of the three values for each of the different breeds is as follows: Havana-3, Dutch-6, Rex-10, English-11, and Himalayan-15. Evidently the disease in the Havana and Dutch groups was far less severe than in the English, Rex, and Himalayan animals. This was so despite the considerable difference in the general character of the dis-

the incidence of edema, metastatic orchitis, and generalized lesions, and for the actual and relative focal distribution of generalized lesions in Table I; the values for the first experiment were considerably higher than for the second. In addition, the average duration of the period of generalized lesion activity was longer in the first experiment (Table II). The mean time of development of the first generalized lesion was

TABLE II

Mean Time of Occurrence of Various Phenomena of Infection Estimated from the Date of Inoculation

Experiment No.	Breed	No. of animals	Primary orchitis	Critical edema	Metastatic orchitis	Generalized lesions		
						First	Mean of all	Last
1	Havana	4	days	days	days	days	days	days
	Dutch	5	21.5	38.8	60.7	59.0	69.6	73.3
	English	5	21.0	27.3	60.0	65.0	68.0	78.4
	Himalayan	5	22.6	33.4	51.8	61.8	68.5	79.6
	Rex	9	17.8	23.5	58.1	67.1	73.9	88.1
2	Havana	3	20.7	27.3	54.0	55.3	70.9	84.7
	Total...	26	20.2	29.3	56.8	63.1	72.4	80.3
	Havana	6	20.7	28.0	70.3	—	—	—
	Dutch	6	19.8	23.3	55.3	73.0	73.0	73.0
	English	6	21.5	26.3	46.4	62.3	59.6	67.3
Total...	Himalayan	6	21.7	40.7	56.8	65.8	68.9	74.8
	Rex	7	17.1	22.0	44.6	58.8	57.8	58.8
Total...		31	20.1	28.3	48.4	63.3	63.7	68.0

63 days in both experiments, but the mean value for the last lesions was 80 days in the first and but 68 days in the second series.

Variations in the severity of experimental syphilis are well recognized, not only in individual animals of any particular group, but also in different groups. Such factors as differences in the quality of the inoculum, the route of inoculation, the season of the year at which the experiments are being conducted, and animal age and sex are known to have an influence on the character of the disease. In the present instance the reason for the mildness of the infection in the second experi-

tain racial characteristics. With some of the older breeds, such as the Dutch, the English, and the Himalayan of these experiments, distinctive racial characters have been developed through many generations, and these breeds should be comparatively homogeneous, functionally as well as physically. With the newer breeds, of which the Havana and the Rex are examples, the situation is different. These breeds are developed from one or more of the older breeds, and different strains or families may be developed by the use of different crosses which would tend to produce variation rather than homogeneity. There is some evidence, however, that even in crosses of this kind there is a tendency to preserve the characteristics of the parental race. Thus, the Havana was derived from the Dutch, and in the main, has been bred to Dutch type; in these experiments Dutch and Havana stand close together. The Rex is derived from the Belgian and here again there is a close similarity in behavior. Still, this does not remove the possibility that there may be marked variations between different lines or families of a given breed or race.

CONCLUSIONS

It is evident that on the basis of the criteria already discussed the Havana and Dutch rabbits of the experiment may be classified as relatively resistant and the English, Himalayan, and Rex rabbits as relatively susceptible to syphilitic infection. The experiments were planned to eliminate all known variables that might influence the course of the syphilitic reaction. The animals were all of approximately the same age, they were caged and fed under uniform conditions, the strain of spirochete employed and the dose and route of inoculation were identical. The only known variable that can account for the observed differences was that of breed. Future experience will show whether the breed differences encountered are constant. Certainly they were constant in the two experiments here reported. Under the conditions of the experiment, therefore, it would seem that the widely divergent reactions are explicable on the basis of variations in breed or race.

These observations emphasize the necessity for an adequate balancing of the breeds of animals selected for syphilitic and similar experiments. Obviously if the Havana rabbits of this report were the ani-

detected earlier and in the English group later than in any of the other breeds.

Critical Edema.—(Table III.) The incidence of edema of the scrotum and inoculated testicle was lower in the Dutch and Rex and higher in the English, Havana, and Himalayan breeds than the combined incidence of the entire group. An estimation of the mean time of occurrence of edema from the date of inoculation indicates that it developed earlier in those breeds that showed the lowest incidence; that is, the Rex and Dutch. Edema developed comparatively late in the Havana, English, and Himalayan groups in which the incidence of this phenomenon was high. If the appearance of edema is reckoned from the time of occurrence of the disease phenomenon immediately preceding it, namely the primary orchitis, the time relationships are somewhat changed. The longest interval between orchitis and edema occurred in the Havana group (13.0 days) and the shortest in the Dutch group (5.7 days). The English, Himalayan, and Rex animals struck a fairly uniform level between these two extremes. These results are suggestive of differences in the reactions of the several breeds.

Metastatic Orchitis.—(Table III.) The incidence of metastatic orchitis in the combined Havana and Dutch groups, 60.0 and 63.6 per cent, was appreciably lower than the combined group mean value of 78.6 per cent. The English, Himalayan, and Rex rabbits showed a higher incidence than the group mean, namely, 90.9, 92.9, and 80.0 per cent respectively. It will be noted that, in the Dutch animals, the low incidence of edema was followed by a low incidence of metastatic orchitis, a result which indicates that a highly efficient protective reaction had been established early in the infection; that is, in association with the primary orchitis. On the other hand, the low incidence of edema in the Rex group was followed by a high incidence of metastatic orchitis, indicating that a comparable defence reaction either had not developed in this breed, or was of brief duration. A comparatively high incidence of edema in the Havana group was followed by the lowest incidence of metastatic orchitis of all the breeds, suggesting that the immunity developed during the critical edema phase was sufficient to affect a partial suppression of the metastatic orchitis. In the English and Himalayan groups, the frequencies of both edema and

larly, the mean number of generalized lesions for each breed group as determined by actual count, that is, the relative focal distribution of generalized lesions, was lower in the Havana (1.6) and Dutch (2.1) groups than in the English (3.5), Himalayan (7.0), and Rex (5.5) groups.

The mean time of appearance of the first generalized lesion in the Havana group was earlier than the appearance of the metastatic orchitis. This overlapping is of importance from the standpoint of resistance since it signifies that the usual orderly sequence of events has been interrupted by the development of a powerful defence reaction.

TABLE IV

Incidence, Focal Distribution, and Mean Time of Occurrence of Generalized Lesions in Combined Experiments

Breed	Incidence	Focal distribution		Time of occurrence				
				From inoculation to			From metastatic orchitis to	
		Actual	Relative	First	Mean of all	Last	First	Last
	<i>per cent</i>			<i>days</i>	<i>days</i>	<i>days</i>	<i>days</i>	<i>days</i>
Havana.....	30.0	5.3	1.6	59.0	69.6	73.3	-1.3	12.7
Dutch.....	54.5	3.8	2.1	66.3	68.8	77.5	2.0	14.8
English.....	72.7	4.9	3.5	62.0	65.2	75.0	12.8	25.8
Himalayan.....	86.7	8.1	7.0	66.6	72.0	83.0	7.4	24.6
Rex.....	70.0	7.9	5.5	57.3	63.4	69.9	11.1	23.6
Total.....	64.9	6.4	4.1	63.2	68.2	77.1	7.6	22.1

In Table IV the interval between the metastatic orchitis and the first generalized lesion is a minus quantity for the Havana group, showing overlapping. In the Dutch group the time interval was only 2 days; that is, although a tendency to overlap was present, it was not quite so marked. The basic tendency of a reaction interval of 1 to 2 weeks between successive stages was maintained by the English, Himalayan, and Rex groups.

The interval between the development of an orchitis of the uninoculated testicle and the appearance of the last generalized lesion is the period during which the disease activity is indicated by a tissue re-

ANIMAL PASSAGE OF PSEUDORABIES VIRUS

The Effect of Serial Passage through Rabbits and Guinea Pigs upon the Pathogenicity of Pseudorabies Virus

The observation that virus from the brain of a guinea pig was innocuous when administered subcutaneously to guinea pigs was made repeatedly with virus that was only 1 or 2 passages removed from rabbits. It seemed of interest, therefore, to determine certainly whether the variations in pathogenicity exhibited by pseudorabies virus of guinea pig and rabbit origin were of a transient nature and induced only for the short time during which it was being adapted to a new host. Consequently, the virus was passed serially through the

TABLE I
Effect of Serial Passage through Rabbits and Guinea Pigs upon the Pathogenicity of Pseudorabies Virus

No. of intracerebral serial passages	No. of animal inoculated subcutaneously with 100 mg. of infected brain, and result
12th rabbit passage—brain Rabbit 118	Rabbit 121—Died 80 hrs. " 122— " 86 "
9th guinea pig passage—brain Guinea Pig 213	Guinea Pig 216—Died 66 hrs. " " 217— " 78 " Rabbit 119—Died 82 hrs. " 120— " 92 " Guinea Pig 214—No illness " " 215— " "

brains of 9 guinea pigs to establish a guinea pig type virus and through the brains of 12 rabbits to establish a rabbit type virus. All of the passage inoculations terminated fatally. The two sets of passages were conducted simultaneously and covered the same total elapsed time. The fact that the disease was more rapidly fatal in rabbits accounted for the greater number of rabbit passages accomplished during the period. The results of tests of the pathogenicity of the two types of virus are outlined in Table I.

The data recorded indicate that prolonged serial passage does not affect the differing pathogenicity of the virus from rabbit and guinea pig brain as determined by subcutaneous injection into guinea pigs 100 mg. amounts.

fective animal due to the fact that a large proportion of the young develop bone deformities which resemble those of rickets. It was mainly for this reason that the Rex, which is of Belgian ancestry, was included in these experiments.

Eye lesions usually develop later than 3 months following inoculation, the time when these experiments were terminated. A certain number, however, did develop during the observation period. It will be seen by referring to Table V that the proportion of eye involvement to all lesions was highest in the Havana group. Since the eye is not protected as fully as other tissues by the general reaction to infection (6), it is not surprising that the Havana animals which de-

TABLE VI

Number and Percentage of Animals with Complete Healing of All Lesions at the Termination of the 3 Months Observation Period—Combined Results

Breed	No. of animals	Complete healing of lesions	
		No.	per cent
Havana.....	10	9	90.0
Dutch.....	11	9	81.8
English.....	11	7	63.6
Rex.....	10	5	50.0
Himalayan.....	15	5	33.3
Total.....	57	35	61.4

veloped a highly efficient reaction as evidenced by the incidence and focal distribution rates of generalized lesions should show a high proportion of eye lesions.

Recovery.—(Table VI.) A final comparison of the behavior of the various breeds in the presence of infection may be made on the basis of the proportion of animals of each breed group that showed complete resolution and healing of all lesions at the termination of the 3 months period of observation. The reaction to the infection in the Havana and Dutch breeds was such that within 3 months after inoculation all lesions were healed in a large proportion of animals. This result, which is in entire harmony with the preceding course of events, is additional evidence that these breeds possess or are capable of develop-

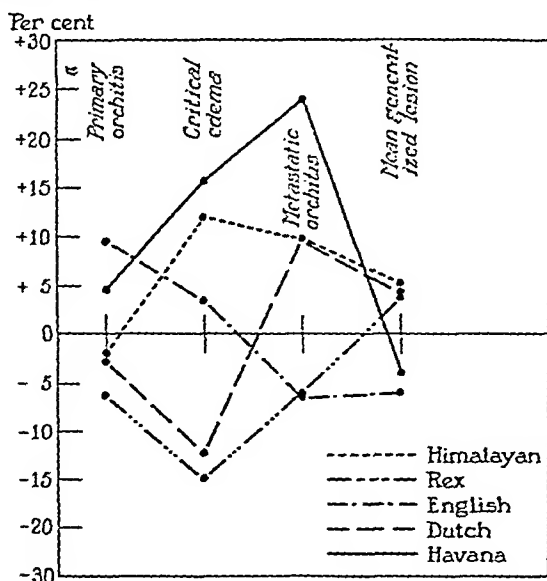
Titration of the Pseudorabies Virus

The experiments recorded above suggested strongly that passage of pseudorabies virus through guinea pigs attenuated it for guinea pigs without apparently affecting its pathogenicity for rabbits. To determine whether factors other than attenuation entered to account for the alteration in pathogenicity of the virus by guinea pig passage, a series of virus titrations was conducted. In all, 4 titration experiments were run, using 4 different samples of rabbit brain virus and 4 of guinea pig brain virus. Since the end-points of the titrations coincided approximately, the results of the experiments have been combined and recorded in Table III. In addition, animals receiving the standard 10 mg. intracerebral or 100 mg. subcutaneous dose of either type of virus during the period of the titration experiments have been included in the table to indicate the uniform nature of the results obtained at these dosages.

In the titration experiments dilutions were arranged in intervals of 10 and no effort was made to obtain more definite end-points. Fresh infected brain was used as the source of virus and the quantity administered was calculated on the basis of wet brain weight. The amounts of virus given intracerebrally were administered in 0.1 cc. quantities of physiological saline, while the subcutaneous doses were given in 1 cc. of physiological saline except in the case of the large amounts of guinea pig brain virus that it was necessary to use in infecting guinea pigs. In this last case infected brain was prepared in a 20 per cent suspension and the amount of this suspension administered determined by the size of dose desired. In a single experiment the type of virus under titration was injected into both guinea pigs and rabbits, so that between the species, the results are comparable. The results of the titrations are recorded collectively in Table III.

As shown in Table III, the minimal fatal dose of intracerebrally administered rabbit brain virus was, within a dilution factor of 10, the same for both rabbits and guinea pigs. The same was true for the guinea pig brain virus except that the fatal dose for both species was 0.1 mg. instead of 0.01 mg. Assuming that the difference in titration end-point was a matter of concentration of virus and not of attenuation for both species by guinea pig passage, it may be concluded that the brain of the rabbit contains roughly ten times as much active virus as does that of the guinea pig. This view is further supported by the fact that while 0.1 mg. of the rabbit brain virus proved fatal for rabbits

several breeds is illustrated by Text-figs. 1 and 2, based on percentage deviations from the mean values of the incidence and time of development of the successive phases of the disease. The percentage deviation figures were in each case obtained from the total mean values of each experiment and then combined as an algebraic sum. This was done in order to eliminate any error that might arise from quantitative differences in the disease picture in the two experiments. Except for one or two minor discrepancies, the relative positions of the different



TEXT-FIG. 2. Time of occurrence of various phenomena of infection. Calculated as combined percentage deviation from mean values in two experiments.

breeds are identical with their relative positions obtained by an analysis based on the mean values for all animals of each breed when the two series are combined and considered as one experiment (Tables III and IV). Text-fig. 3 illustrates graphically the combined values for the different breeds with respect to the incidence of metastatic orchitis, the incidence of generalized lesions, the relative focal distribution of generalized lesions, and the proportion of animals of each breed with complete healing of all lesions at the end of the experiment. These text-figures represent in striking manner the difference in behavior of the several breeds of rabbits to the syphilitic infection.

attenuation of the virus on passage through guinea pigs, two factors besides attenuation enter to make the alteration in pathogenicity appear greater than it is. The first of these has to do with the presence in rabbit brain of approximately ten times more titratable virus than in guinea pig brain. The second concerns the observation that the guinea pig is approximately 100 times more naturally resistant to pseudorabies virus than is the rabbit.

Since both of these species differences are in the same direction, the question arises as to whether they alone are sufficient to account for what, in the beginning, appeared to be an attenuation of virus by the guinea pig. A tenfold greater concentration of virus in rabbit brain together with a hundredfold greater natural resistance on the part of guinea pigs to subcutaneous infection makes a thousandfold difference with regard to the two species that cannot be attributed to attenuation as it is generally understood. Taking this difference into consideration and calculating on the basis of the known minimal fatal doses of both types of virus for rabbits subcutaneously, the subcutaneous minimal fatal dose of guinea pig brain virus for guinea pigs should have been in the neighborhood of 100 mg. Since titration indicates that it was considerably higher than this, it is apparent that guinea pig passage exerts some attenuating influence on the pseudorabies virus for that species. The fact that the attenuation was evident upon subcutaneous infection and not when the guinea pigs were intracerebrally infected suggests that the explanation for the phenomenon is physical or mechanical.

That the attenuation was not due to an inhibiting substance in either normal or infected guinea pig brain was indicated by a series of experiments that may be briefly stated here. Normal guinea pig brain mixed in 200 mg. amounts with rabbit brain virus in amounts ranging from 10 to 200 mg. failed to prevent a fatal infection when the mixture was inoculated subcutaneously into guinea pigs nor did it lengthen the period of survival of the animals. In like manner, guinea pig brain virus rendered non-infective by heating to 55° and 60°C. for 1/2 hour, when mixed in 200 mg. amounts with rabbit brain virus in 100 mg. amounts, failed to prevent a fatal infection or to lengthen the period of survival when inoculated subcutaneously into guinea pigs.

se in the two experiments. If in the identical manner a numerical value is assigned for the same three criteria of infection in each experiment, as they appear in Table I, the sum of these values for each breed group is as follows: for Experiment 1: Havana-4, Dutch-6, English-9, Himalayan-9, Rex-11; and for Experiment 2: Havana-3, Dutch-6, English-12, Himalayan-14, Rex-10. It is seen that despite the severity of the disease in the first experiment and its mildness in the second which has already been discussed, the Havana and Dutch groups have the lowest values and the English, Rex, and Himalayan the highest in both experiments. In terms of focal distribution of generalized lesions, the Rex groups of the two experiments were not comparable. In the first series the focal distribution values were the highest of the five breeds, but in the second experiment they occupied an intermediate position, being exceeded by the Himalayan and English groups. With regard to this discrepancy, it is important to note that Rex rabbits are extremely susceptible to the exigencies of cage life. In the second experiment the general condition of these animals was not comparable to that of the other breeds. They lost weight, the condition of the fur became poor, and several developed snuffles. Long experience in this laboratory has indicated that the optimum reaction develops in animals of good physical condition. In animals showing evidence of nutritional deficiency, the response to infection is minimal or subminimal. The fact that the Rex animals of the second experiment developed only a few generalized lesions may be explained at least in part by their impaired physical state. It is possible also that a contributory factor was the disproportion in numbers, three animals in the first experiment and seven in the second.

Animal Material.—It should be emphasized that the results here reported are applicable only to the particular family or families of each standard breed which are bred and studied in this laboratory. Similar experiments on animals of the same breeds obtained from other sources might produce different results since it is not yet known whether the families of the different breeds here employed represent a fair sample of the particular breed.

In this connection, it should be pointed out that one of the objects in the adoption of standards of perfection by fanciers and breeders associations is to encourage the development and maintenance of cer-

mals used for some experimental procedure which employed the Himalayans as control material, or *vice versa*, the resulting marked difference in the course of disease in the two groups could not be properly attributed to the experimental procedure. It is also conceivable that differences in the disease, either qualitative or quantitative occurring in different laboratories and countries, might be caused by differences in the breed of rabbits employed.

The demonstration of distinct racial or familial differences in the response of experimental animals to syphilitic infection is moreover of interest on account of the support which it lends to the view that in man there are comparable racial differences in susceptibility to syphilis.

SUMMARY

A comparative study was made of the character and severity of the infection produced by *Tr. pallidum* in groups of standard bred Havana, Dutch, English, Himalayan, and Rex rabbits. All known variables that might influence the course of the disease were common to the different breed groups.

On the basis of certain clinical data selected as criteria for the determination of variations in the response to syphilitic infection, it was found that the Havana and Dutch animals were resistant while the English, Himalayan, and Rex rabbits were susceptible.

From these results, it appears that breed, race, or family is a factor which significantly influences the response to infection with *Tr. pallidum*.

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ideal condition can be imagined for the entrance of a neurotropic virus deposited on the surface.

Assuming that the nasal mucosa, or, more correctly, the olfactory cells and nerves, constitute the normal gateway through which poliomyelitis virus gains access to the central nervous system in man, the subsequent pathways by which it reaches the site of its maximum effects, the anterior horns of the spinal cord, still remain unknown. Only a few recorded attempts have been made to determine experimentally the various localizations of virus during the incubation period after intranasal inoculation, most of which have given negative results and apparently discouraged further study.¹ In 1912, Flexner and Clark (7) showed that 48 hours after swabbing the nasal mucosa of a monkey with virus, the olfactory bulb contained a demonstrable amount of virus, while none could be detected in the medulla or in the spinal cord. The earlier demonstration by Landsteiner and Levaditi (8) of virus in the olfactory bulbs 11 days after nasal inoculation (submucous injection) did not include the study of other possible points of infection in the central nervous system. In 1920, Flexner and Amoss (9) reported an experiment in which they left a cotton pledget soaked with virus in the nose of a monkey for 24 hours. The animal was sacrificed 88 hours later and the olfactory lobes, postrolandic convolutions, medulla, cervical cord and lumbar cord were tested for the presence of virus, all giving negative results. In 1930, Fairbrother and Hurst (10) reported the results of four experiments with intranasal inoculation, in two of which virus was not detected anywhere in the central nervous system. In the other two, positive results were obtained. In one animal examined 12 days after inoculation, virus was found in the homolateral olfactory lobe and in the cervical cord; not in the left anterior frontal cortex, right olfactory lobe, lumbar cord

¹ The essential difficulty has been that experimental infection by the nose was not regularly obtained by the methods generally used until within the last 2 or 3 years. Howitt (5) estimates that only 53 per cent of intranasal inoculations are successful. The percentage is certainly less than this at times. Flexner (6) has recently been able to obtain much more uniform takes by instillations repeated over a period of several days. In our own work, no progress could be made for several months until the technique was modified (or the conditions changed spontaneously for unknown reasons), after which we had almost no failures and were able to go ahead with the present study.

MODIFICATION OF THE PATHOGENICITY OF PSEUDORABIES VIRUS BY ANIMAL PASSAGE

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INTRODUCTION

The identity of "mad itch" with the disease known in Europe as pseudorabies was suggested in an earlier paper (1) and more recently confirmed immunologically by the method of cross-neutralization (2). The use of the picturesque term "mad itch," while highly descriptive of the disease to which it has been applied in this country, must therefore give way to the earlier and somewhat misleading term pseudorabies. The etiological agent, which differs in certain ways (1) from the original Hungarian strain of pseudorabies virus (3) obtained from Aujeszky, with which it was compared, will hereafter be referred to as pseudorabies virus (Iowa strain). Only the Iowa strain has been employed in the present work.

EXPERIMENTAL

In our experimental work with pseudorabies virus it has been consistently observed that virus obtained from the brain of an intracerebrally infected rabbit differed in infectivity from that similarly obtained from the brain of an intracerebrally infected guinea pig. When a standard subcutaneous injection of 1 cc. of a 10 per cent suspension of infected brain (100 mg.) was administered, the virus from rabbits was regularly fatal to both rabbits and guinea pigs, while that from guinea pigs was pathogenic for rabbits only. Intracerebral inoculations, however, of 10 mg. doses of either type of virus were regularly fatal to both species. Further experiments, to be reported here, were conducted to determine the nature of the change induced in the pseudorabies virus by guinea pig passage.

mately pH 5.0,² with the animal's head in the dependent position; immediately thereafter 0.5 cc. of the 10 per cent virus suspension was dropped into the nose. 3 hours later and, again, 6 hours later, virus suspension in the same amount was dropped into the left naris. The acid wash was not repeated. No traumatization was involved in any part of the procedure.

23 of 26 animals³ thus treated and permitted to survive developed typical poliomyelitis. The average period of incubation was 8.2 days; the shortest individual period was 7 days and the longest, 10 days. The availability of a method giving such almost uniformly positive results and permitting the dating of infection from a single day was essential to the study of the problem.

Animals thus treated were killed by ether on the 3rd, 4th, 5th, 6th and 7th days of incubation, respectively. They were immediately dissected under strictly aseptic precautions and the brain, and the spinal cord with its ganglia, removed. The brain was divided into two lateral halves, with particular care to divide the brain stem and cerebellum in the midline. Pieces were then excised from the various areas to be described. All instruments were sterilized before being used for another excision. When specimens were to be taken from closely adjoining areas such as the hypothalamus, thalamus and midbrain, the precaution was taken of leaving a bridge of tissue between the excised areas. This precaution could not, however, be taken in separating the anterior and posterior halves of the cord.

The selection of areas for testing was primarily based on a study of the olfactory tracts and their connections, discussed elsewhere (2). The reader is reminded that from the olfactory bulb the main tract connections are (a) with the hypothalamus, thalamus and midbrain, and (b) with the olfactory cortex (hippocampus, uncus, gyrus fornicatus, etc.), and thence with the hypothalamus, thalamus and midbrain. From the hypothalamus down to the medulla is a tract or series of tracts, in the formatio reticularis which appears to have special importance in poliomyelitis, since it has been found to be frequently and heavily involved in the human pathology (14). Connections with the spinal cord and its ganglia are traceable through the great sensory tracts, especially the spinothalamic from the thalamus, to the posterior horns and ganglia; and through various motor pathways having relays in the midbrain and medulla, directly to the anterior horns of the cord. For certain reasons, mainly clinical, discussed in

² With reference to the value of this method, which was introduced by Schultz and Gebhardt at the Department of Bacteriology of Stanford University, Schultz in a recent personal communication to me states that the results thus far obtained indicate that the incidence of takes is somewhat increased by preliminary nasal washes with acid phosphate solutions. A note on the subject is being submitted to the *Proceedings of the Society for Experimental Biology and Medicine* for publication.

³ These were used in other experiments by Schultz and Gebhardt.

The Alteration of the Pathogenicity of Pseudorabies Virus in a Single Passage

To determine whether the change from rabbit type to guinea pig type virus could be accomplished in a single passage, experiments like those recorded in Table II were conducted.

In this experiment virus from a rabbit, pathogenic for guinea pigs when administered subcutaneously, was, by 1 cerebral guinea pig passage, rendered non-pathogenic for guinea pigs on subcutaneous

TABLE II

Alteration of the Pathogenicity of Pseudorabies Virus in Single Passage Intervals

Passage	Source of virus	No. of animal inoculated, route and result
1st	Brain Rabbit 140	Guinea Pig 227 Sc—Died 58 hrs.
		" " 228 "— " 58 "
		" " 223 Ic— " 44 "
		" " 224 "— " 49 "
2nd	Combined brains of Guinea Pigs 223 and 224	Guinea Pig 231 Sc—No illness
		" " 232 "— " "
		" " 229 Ic—Died 54 hrs.
		" " 230 "— " 54 "
3rd	Brain Guinea Pig 230	Rabbit 132 "—Died 46 "
4th	Brain Rabbit 132	Guinea Pig 237 Sc—Died 67 "
		" " 238 "— " 67 "

Sc = subcutaneously. Ic = intracerebrally.

inoculation. 1 subsequent passage of this guinea pig virus through the brain of a rabbit restored its full pathogenicity for guinea pigs by subcutaneous inoculation.

That the alteration in pathogenicity of pseudorabies virus was not contingent upon a particular route of inoculation was indicated by the fact that the lung and brain of an intranasally inoculated guinea pig and the local subcutaneous lesion of a subcutaneously inoculated guinea pig contained virus which, while fully pathogenic for rabbits when administered subcutaneously, was completely innocuous for guinea pigs by the same route.

EXPERIMENTAL RESULTS

Three entirely negative experiments should be mentioned, in which the experimental conditions were alike save in respect to the day on which the animals were sacrificed. Three instillations were made at 3 hour intervals in the left naris.

Monkey F78-1 was killed 3 days after virus instillation: no virus was detected in the left olfactory bulb, the left thalamus, the medulla, the cervical and lumbar cord (pooled) nor in the pooled cervical and lumbar ganglia. Those were the only places tested. A control animal treated in exactly the same way, on the same day and with the same suspension of virus, developed typical poliomyelitis on the 10th day. In these experiments only 0.25 cc. of suspension was instilled each time. 0.5 cc. was used in the other experiments. It is, of course, possible that Monkey F78-1 would have succumbed to the disease and that the virus had not yet reached the olfactory bulb in sufficient concentration on the 3rd day of incubation to permit detection.

Monkey F85-1 was killed 5 days after intranasal inoculation. No virus was found in the left olfactory bulb, the left hypothalamus, the left thalamus nor in the medulla (both sides pooled). These were the only places tested.

Monkey F80-4 was killed 6 days after intranasal inoculation. No virus was detected in the left olfactory bulb, the left hypothalamus nor in the medulla (both sides pooled). These were the only places tested.

In the last two experiments it seems probable that no infection had occurred.

PROTOCOLS

Series A. 4th Day of Incubation

Monkey F80-3.—Oct. 21, 1932: 3 instillations in left naris, of 0.5 cc. of a 10 per cent suspension of MV virus (3 cords pooled), at 3 hour intervals. Killed Oct. 25. Specimens ground and diluted to approximate 10 per cent suspension in normal saline solution.

Subinoculations

Left Olfactory Bulb.—Monkey F80-5, inoculated Oct. 26; 2.0 cc. Oct. 30, poliomyelitis. Oct. 31, complete flaccid paralysis; killed.

Hippocampus, Right and Left, Pooled.—Monkey F81-1, inoculated Nov. 8; 2.0 cc. Remained well.

Left Hypothalamus.—Monkey F80-6, inoculated Oct. 26; 2.0 cc. Remained well.

Medulla, Right and Left Sides, Pooled.—Monkey F80-7, inoculated Oct. 26; 2.0 cc. Remained well. Susceptible in later experiment (F84-0).

when administered subcutaneously, 1 mg. of the guinea pig brain virus was required to induce a similar effect.

Consideration of the data in Table III indicates further that the guinea pig possesses roughly a hundred times greater natural resistance to subcutaneously administered pseudorabies virus than does the rabbit, because, in titrating rabbit brain virus, 0.1 mg. was found to

TABLE III
Titration of Rabbit and Guinea Pig Types of Pseudorabies Virus

Dosage	Animals receiving rabbit brain virus								Animals receiving guinea pig brain virus							
	Guinea pigs Ic*		Rabbits Ic		Guinea pigs Sc*		Rabbits Sc		Guinea pigs Ic		Rabbits Ic		Guinea pigs Sc		Rabbits Sc	
	No. inoculated	No. died	No. inoculated	No. died	No. inoculated	No. died	No. inoculated	No. died	No. inoculated	No. died	No. inoculated	No. died	No. inoculated	No. died	No. inoculated	No. died
mg.																
0.0001	4	0	4	0												
0.001	6	2	6	2			1	0	3	0	3	0				
0.01	6	6	6	6	2	0	5	0	4	0	4	2			2	0
0.1	2	2	2	2	6	0	5	5	4	3	4	4			4	1
1	1	1	1	1	7	3	3	3	1	1	1	1			4	4
10	11	11	3	3	7	5			13	13					1	1
100					43	43	22	22					53	3	3	3
200													5	0		
400													8	2		
600													6	2		
800													6	4		
1000													5	4		

* Ic = intracerebrally. Sc = subcutaneously.

be fatal to rabbits subcutaneously while 10 mg. of the same virus was required to kill guinea pigs, at all regularly, by the same route.

DISCUSSION

In attempting to determine the factors underlying an alteration in the pathogenic properties of pseudorabies virus by guinea pig passage, we have resorted to titration of rabbit and guinea pig types of virus. These titration experiments indicate that, in addition to some apparent

LOCALIZATIONS OF POLIOMYELITIS VIRUS

Left Hypothalamus.—Monkey F88-7, inoculated Dec. 21, 1932; 1.5 cc. 29, poliomyelitis. Dec. 31, complete paralysis. Jan. 3, very low; killed.
Thalamus, Right and Left, Pooled.—Monkey F88-9, inoculated Jan. 5, 1933; 2.0 cc. Remained well.
Midbrain, Right and Left Sides, Pooled.—Monkey F92-8, inoculated Jan. 1933; 1.5 cc. Remained well.

TABLE II
Monkey F80-4. 5th Day of Incubation. Positive Results. Periods after Intraneural Inoculation When Typical Symptoms Appeared, and When Paralysis Became Practically Complete

	Day first symptoms	Day complete paralysis
Left olfactory bulb.....	6	8
Right olfactory bulb.....	7	9
Left hypothalamus.....	8	10
Medulla.....	6	10

TABLE III
5th Day of Incubation. Monkey F88-4. Positive and Negative Results

Side tested.....	Left	Right	Both
Olfactory bulb.....	+ (6)	+ (7+)	
Cortex.....			
Hippocampus.....			0
Interbrain.....			
Hypothalamus.....	+ (8)	—	
Thalamus.....			0
Midbrain.....			0
Pons-medulla.....			+ (6)
Cerebellum.....			0
Cord, cervical.....			0

Medulla, Right and Left Sides, Pooled.—Monkey F88-8, inoculated Dec. 21, 1932; 1.5 cc. Dec. 27, poliomyelitis. Dec. 29, paralysis, right leg. Dec. 31, complete paralysis; killed.
Cerebellum, Right and Left, Pooled.—Monkey F89-2, inoculated Jan. 5, 1933; 2.0 cc. Remained well.
Spinal Cord, Cervical.—Monkey F88-9, inoculated Dec. 21, 1932; 1.5 cc. Remained well.

Summary.—Virus detected in left olfactory bulb, in left hypothalamus, in left and right thalami, in midbrain, in medulla; highest concentration apparently in left hypothalamus, and next highest in left olfactory bulb. Concentrations approximately equal in the thalamus (right and left), midbrain and medulla.

TABLE IV
6th Day of Incubation. Monkey F83-4. Positive Results

	Day first symptoms	Day complete paralysis
Left olfactory bulb.....	6	9
Left hypothalamus.....	5	7
Left thalamus.....	9	10
Right thalamus.....	8	11
Midbrain.....	8	9
Medulla.....	9	10

TABLE V
6th Day of Incubation. Monkey F83-4. Positive and Negative Results

Side tested.....	Left	Right	Both
Olfactory bulb.....	+(6)	0	
Cortex			
Hippocampus.....			0
Precentral gyrus.....			0
Postcentral gyrus.....			0
Interbrain			
Hypothalamus.....	+(5)	0	
Thalamus.....	+(9)	+(8)	
Caudate nucleus.....	0		
Midbrain.....			+(8)
Pons-medulla.....			+(9)
Cerebellum.....			0
Cord			
Cervical.....			0
Lumbar.....			0
Dorsal root ganglia.....			0

Series D. 7th Day of Incubation

Monkey F86-3.—Dec. 6, 1932: 3 instillations in left naris of 0.5 cc. of a 10 per cent suspension of MV virus (5 cords pooled) at 3 hour intervals. Dec. 13, temperature 105.4°; no other signs of poliomyelitis; killed.

LOCALIZATIONS OF THE VIRUS OF POLIOMYELITIS IN THE CENTRAL NERVOUS SYSTEM DURING THE PREPARALYTIC PERIOD, AFTER INTRA- NASAL INSTILLATION*

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The view that the virus of poliomyelitis enters the body through the nasal mucous membrane, first suggested by Flexner (1) in 1910 and consistently advocated by him and his associates since that time, appears now to be widely accepted. It is based on an impressive body of experimental evidence (2) showing that the nasal mucosa is unique among the body surfaces in permitting the ingress of virus without preliminary trauma. The relationship of this phenomenon to certain anatomical arrangements in the nose has been almost overlooked. Flexner in 1912 (3) commented on the fact that "the small olfactory filaments are advantageously placed to act as the means of transportation" (of the virus). It was not, however, until the discovery that poliomyelitis virus is propagated through the nerve cells and their axons, rather than through the blood, lymph or cerebrospinal fluid, that the nervous anatomy of the region took on unique significance in relation to poliomyelitis. Faber (2) has called attention to the importance of the unbroken connection of the olfactory nerves between the very surface of the nasal mucosa and the central nervous system proper. Hopkins (4) has shown that the terminal processes (olfactory hairs) of the olfactory cells are exposed to the air in the upper part of the nasal cavity, and the afferent axons of these cells pass through the olfactory nerves directly into the olfactory bulbs. No similar arrangement exists elsewhere on the body and no more

* This study was aided in large part by a grant from the Rockefeller Fluid Research Fund of the School of Medicine of Stanford University.

Dorsal Root Ganglia, Right.—Monkey F88-1, inoculated Dec. 14, 1932; 1.5 cc. Dec. 23, poliomyelitis. Dec. 24, paralysis both arms. Dec. 25, died.

TABLE VI
Monkey F86-3. Positive results

	Day first symptoms	Day complete paralysis
Left olfactory bulb.....	6	9
Right olfactory bulb.....	7	9
Hippocampus.....	12	14
Right thalamus.....	15	16
Midbrain.....	8	10
Medulla.....	6	8
Cord		
Cervical, anterior half.....	7	10
Cervical, posterior half.....	7	10
Lumbar, anterior half.....	10	11
Cervical and lumbar, posterior half.....	7	8
Dorsal ganglia, left.....	12	14
Dorsal ganglia, right.....	9	10+

TABLE VII
7th Day of Incubation. Monkey F86-3. Positive and Negative Results

Side tested.....	Left	Right	Both
Olfactory bulb.....	+(6)	+(7)	
Cortex			
Hippocampus.....			+(12)
Pre-, postcentral gyri.....			0
Interbrain			
Hypothalamus.....	0	0	
Thalamus.....	0	+(15)	
Midbrain.....			+(8)
Pons-medulla.....			+(6)
Cerebellum.....			0
Cord			
Cervical anterior half.....			+(8)
Cervical posterior half.....			+(8)
Lumbar anterior half.....			+(10)
Lumbar posterior half.....			—
Cervical and lumbar, posterior half.....			+(7)
Lumbosacral.....			0
Dorsal root ganglia, cervical and lumbar.....	+(12)	+(9)	

or cerebrospinal fluid. In the other, examined 11 days after inoculation, virus was found in the cervical and lumbar cord; not in the left anterior frontal cortex. In both these experiments, however, the disease had progressed to the point of paralysis and the results give no clear indication of the localizations of infection during the incubation period proper. The histological study of the animals showed lesions particularly marked in the nasal cortex of the homolateral hemisphere, in the amygdaloid nucleus (one of the olfactory relays) and the cornu Ammonis (hippocampus); less in the homolateral thalamus and globus pallidus; none in the putamen and caudate nucleus. We find no other comparable experiments in the literature.

The experiments of Flexner and Clark, therefore, appear to be the only ones affording any positive information on the localization of virus during the preparalytic period of infection in animals intranasally infected (the presumptively normal route), and these experiments show only its very earliest localization, in the olfactory bulbs.

The demonstration of the axonal mode of propagation by Fairbrother and Hurst (10), since confirmed by Jungeblut and Spring (11), and by later experiments of Hurst (12) justifies the expectation that the natural route followed by virus from this initial point of attack should be along the tracts leading out from the olfactory bulbs and along others with which they connect. The present study is based on such an assumption and the experiments have been planned in accordance with it. We have attempted to map the distribution of virus in the central nervous system on successive days of the incubation period; to determine which areas are spared as well as those which are infected, and thus to discover whether or not a definite and continuous route of propagation can be discerned, leading from the primary site of implantation in the central nervous system down to the spinal cord.

Methods

Macacus rhesus monkeys were used in all the experiments. The source of virus was a 10 per cent pooled suspension of cord, or cord and medulla, from 3 to 5 monkeys infected with the MV strain, each lot having been tested in the usual manner for virulence. For the present series and the controls a uniform technique of inoculation was followed. The left naris was always used, and an effort was made to confine the preliminary treatment as well as the inoculum to this side. First the naris was flooded with a slightly acid buffer solution at approxi-

Summary.—Virus detected in both olfactory bulbs, in hippocampal cortex (small amounts), in right thalamus (small amounts), in mid-brain, in medulla, in both anterior and posterior halves of cervical cord, in anterior half of lumbar cord (in smaller amounts than in cervical), in dorsal root ganglia both right and left (more in right). The highest concentrations were found in the left olfactory bulb and in the medulla, and a little less in the specimen from the posterior half of the cervical and lumbar cord; other high concentrations were found in the right olfactory bulb, and the anterior and posterior half of the cervical cord.

DISCUSSION

After deposition on the nasal mucosa the virus of poliomyelitis apparently requires a period of 2 to 4 days for penetration into the olfactory cells, ascent through the olfactory nerves into the olfactory bulb and multiplication therein to amounts sufficient for detection. That this is the actual pathway of ascent is shown by the occurrence of virus in the olfactory bulb (homolateral side with the naris inoculated) before it could be detected in any other of the nervous areas examined; by its absence from the outgoing olfactory pathways (hypothalamus, hippocampus), and by the extraordinarily high concentration of virus in the olfactory bulb. So sharp a localization and heavy concentration of virus cannot be interpreted otherwise than by axonal passage through the ascending olfactory neurons, since the other alternative—*ascent by the perineural lymphatics into the subarachnoid space—must necessarily lead to much wider diffusion of the virus.* *The olfactory bulb, therefore, is to be regarded as the initial focus of poliomyelitic infection within the central nervous system, directly infected through the olfactory nerves from the nasal mucosa.* By the 4th day of the incubation period it has become a heavily charged reservoir joined by an elaborate and extensive system of communications with other parts of the central nervous system through which infection can be rapidly discharged and distributed.

Throughout the incubation period the olfactory bulb continues to be heavily infected.

On the 5th day, virus is found, as yet in smaller amounts than in the primary focus, in one of the primary relays of the olfactory tracts.

another paper (2), it has been surmised that the chief pathway of infection in man is along the great spinothalamic sensory pathway to the posterior horns and thence to both the dorsal root ganglia and anterior horns.

On the basis of the hypothesis just outlined, specimens were removed from the following areas: the olfactory bulbs; the hypothalamic area; the thalami; the mid-brain; the medulla with the lower half of the pons (designated medulla in the protocols); the spinal cord at different levels, in some instances separating the anterior and posterior portions approximately at a line just behind the posterior edge of the grey commissure (in this case it was impossible because of the smallness of the cord to leave a space between the excised portions; the two sections were therefore immediately contiguous); the dorsal root ganglia, right and left separately. While the neopallial cortex (the portion exclusive of the olfactory cortex) and the cerebellum are not in the direct line of connection with the olfactory tracts, portions of these were also removed: the frontal pole of the frontal lobe; the precentral (prerolandic) gyrus of the frontal lobe (cortical motor area); the postcentral gyrus of the parietal lobe (cortical sensory area, connected directly with the thalamus); the lateral lobes and vermis of the cerebellum.

The portions removed were weighed (or with very small specimens the weight was estimated); physiological salt solution was added sufficient to make a 10 per cent suspension; the mixtures ground in mortars for $\frac{1}{2}$ hour, according to the method of Schultz and Banham (13), and stored until used. In testing for virus, measured amounts of the suspensions (1.5 and 2.0 cc.) were injected intracerebrally according to standard technique. The first observed typical symptoms, usually preparalytic (tremor, nervousness, apprehensiveness, occasionally local weakness) have been used to date the time of onset of the disease. In the protocols the designation poliomyelitis has been used for the appearance of these first typical symptoms and should be so interpreted. The time when paralysis became complete or nearly so has also been noted in the protocols. The amount of virus in a given suspension is assumed to be in rough inverse proportion to the time of onset and of complete paralysis. For this reason, a tabulation of these data is given for each of the series of protocols.

Many specimens were examined histologically throughout the study and the typical changes of poliomyelitis found in all instances. It is hoped that at a later time the distribution of lesions in the central nervous system on various days of the incubation period after intranasal inoculation can be studied. The present study deals only with the distribution of virus in amounts capable of detection by subinoculation. It should, of course, be recognized that virus may have been present in smaller amounts than this in other areas than those giving positive subinoculation tests.

In several instances stated in the protocols animals used for subinoculation and found negative were later found to be susceptible to poliomyelitis.

No instance of secondary infection—brain abscess, meningitis, etc.—referable to contamination of the inocula, occurred.

The experiments of the 6th day furnish additional information. At this time, it will be noted that virus was again found in the left olfactory bulb (same side as the inoculation), as it was on every day from the 4th to the 7th inclusive. Its absence from the right side (opposite side from the inoculation) in this experiment, and the smaller amounts on this side than on the left in the 5 and 7 day experiments have an obvious significance. The very high concentration of virus in the left (homolateral) hypothalamus (5 day incubation) is in good agreement with the postulated pathway of infection. The two thalami now show a moderate degree of infection, approximately equal to that also found in the midbrain and medulla. No virus was detected anywhere in the cerebral cortex, left caudate nucleus, contralateral hypothalamus, cerebellum, cervical or lumbar cord or intervertebral ganglia. The appearance of virus in previously uninfected areas—the thalamus and midbrain—would appear to indicate that infection was now following a separate channel from that noted on the 5th day (hypothalamus to medulla). On the basis of the known anatomy, this may have been through the mammillothalamic tract (bundle of Vicq d'Azyr) from the hypothalamus to the anterior nuclei of the thalamus (and the commissural connections between the thalami), and the mammillotegmental tract from the hypothalamus to the midbrain. The presence of virus in the midbrain and its absence in all experiments from the cerebellum are suggestive of a decidedly restricted localization within the relatively small mesencephalic area, since they apparently indicate that the red nucleus—a relay in the cerebellar pathways and a close neighbor of Gudden's nucleus and of the hypothalamus, also—is not involved.

The complete absence of virus in the spinal cord even in the cervical region, on both the 5th and 6th days when it is present in the medulla in fairly high concentration, is extremely interesting, suggesting that the infected areas in the medulla are perhaps not the main source of infection of the spinal cord. The inference was elsewhere made (2), based on considerations of anatomy, pathology and symptomatology, that the main route of invasion of the spinal cord was more probably from the thalamus than from the medulla, and this view is apparently supported by the present experimental observations.

On the 7th day virus is found abundantly and extensively in the

Lumbar and Cervical Cord and Dorsal Root Ganglia, Pooled.—Monkey F81-2, inoculated Nov. 8; 2.0 cc. Remained well.

Summary.—Virus detected in left olfactory bulb only, and in extremely high concentration. The first signs of poliomyelitis in the test monkey appeared on the 4th day, and paralysis was complete on the 5th.

Series B. 5th Day of Incubation

Monkey F88-4. Dec. 15, 1932: 3 instillations in left naris, each of 0.5 cc. of a 10 per cent suspension of MV virus (pooled), adjusted to pH 7.0, at 3 hour inter-

TABLE I
4th Day of Incubation. Monkey F80-3. Positive and Negative Results

Side tested.....	Left	Right	Both
Olfactory bulb.....	†(4)	—	0
Cortex, hippocampus.....			
Interbrain			
Hypothalamus.....	0	—	
Thalamus.....	—	—	
Midbrain.....	—	—	
Pons-medulla.....			0
Cord.....			{0
Dorsal ganglia.....			{0

†: tested by intracerebral inoculation and found positive by development of typical poliomyelitis in test animal; the first definite signs appearing on day after inoculation indicated by figure in parentheses. 0: tested and found negative, test animal remaining well. —: not tested. "Both" indicates that the specimens from the two sides were pooled and tested together.

vals. Dec. 19, temperature 105.6°; no other symptoms. Killed Dec. 20, 1932. Specimens ground and diluted to approximate 10 per cent suspension in normal saline solution.

Subinoculations

Left Olfactory Bulb.—Monkey F88-6, inoculated Dec. 21; 1.5 cc. Dec. 27, poliomyelitis. Dec. 29, complete paralysis. Dec. 30, very low; killed.

Right Olfactory Bulb.—Monkey F93-1, inoculated Jan. 28, 1933; 1.5 cc. Feb. 4, tremors. Feb. 6, complete paralysis; killed.

Hippocampus, Right and Left, Pooled.—Monkey F89-1, inoculated Jan. 5, 1933; 2.0 cc. Remained well.

areas of cord for the tests. Unfortunately, too, one test could not be made that might have been critical—of the posterior half of the lumbar cord. The experiments actually made, however, show definitely that the posterior half is at least as heavily infected as the anterior at the earliest moment when virus can be detected anywhere in the cord. In the cervical region the two halves appear to be equally infected, since the test animals showed the first signs and developed paralysis at almost exactly the same time. In the anterior lumbar cord, the concentration was less than in the cervical cord, and no virus at all was detected in the lumbosacral segment. The highest concentration found anywhere in the spinal cord was in a pooled specimen of the posterior halves of cervical and lumbar segments. Unfortunately, no note was made of the exact levels from which the pieces were removed (the upper segment was well separated from the medulla) and we are therefore unable to state the point where maximum concentration occurred, except that it was outside the cervical area separately tested (Monkey F92-4).

The presence of virus in the dorsal root ganglia in somewhat smaller amounts than in the cord itself and the definitely larger amount in the contralateral (right) side from the original inoculation afford further evidence of involvement of the sensory tracts, and also suggest propagation along decussating pathways.

The experiments with tissues from the spinal cord therefore indicate invasion of both the anterior and posterior horns shortly (approximately 1 day) before paralysis was to have been expected. About 1 day earlier (6th) in the incubation period no virus was demonstrable anywhere in the cord or its ganglia. On the 7th day it was abundantly present in the cervical segments and in smaller amounts also in the lumbar. The maximum concentration in the cord was found in the posterior half (presumably in the posterior horns). We hope to investigate the localizations in the cord in greater detail in the near future.

SUMMARY

The results of our investigation indicate that in experimental poliomyelitis produced by intranasal inoculation it is possible to trace, always through the nervous system, the descending routes of infec-

Summary.—Virus detected in left and right olfactory bulbs, in left hypothalamus and in medulla; highest concentration apparently in left olfactory bulb.

Series C. 6th Day of Incubation

Monkey F83-4.—Nov. 9, 1932: 3 instillations in left naris, each of 0.5 cc. of a 10 per cent suspension of MV virus (cord and medulla of 6 monkeys, pooled), at 3 hour intervals.

Subinoculations

Left Olfactory Bulb.—Monkey F83-6, inoculated Nov. 16, 1932; 1.5 cc. Nov. 22, poliomyelitis. Nov. 25, complete paralysis. Nov. 26, killed.

Right Olfactory Bulb.—Monkey F85-0, inoculated Nov. 26, 1932; 2.0 cc. Remained well.

Hippocampus, Right and Left, Pooled.—Monkey F83-9, inoculated Nov. 16, 1932; 1.5 cc. Remained well.

Precentral Gyri, Right and Left, Pooled.—Monkey F85-2, inoculated Nov. 29, 1932; 1.5 cc. Remained well.

Postcentral Gyri, Right and Left, Pooled.—Monkey F85-3, inoculated Nov. 29, 1932. Remained well.

Left Hypothalamus.—Monkey F83-8, inoculated Nov. 16, 1932; 1.5 cc. Nov. 21, poliomyelitis. Nov. 22, partial paralysis both arms. Nov. 23, complete paralysis; died.

Right Hypothalamus.—Monkey F88-2, inoculated Dec. 14, 1932; 1.5 cc. Remained well.

Left Thalamus.—Monkey F83-7, inoculated Nov. 16, 1932; 1.5 cc. Nov. 25, paralysis, both arms. Nov. 26, complete paralysis. Dec. 3, killed.

Right Thalamus.—Monkey F88-3, inoculated Dec. 14; 1.5 cc. Dec. 22, poliomyelitis. Dec. 25, complete paralysis. Dec. 28, died.

Left Caudate Nucleus.—Monkey F84-1, inoculated Nov. 16, 1932; 1.5 cc. Remained well.

Midbrain, Right and Left, Pooled.—Monkey F92-9, inoculated Jan. 23, 1933; 1.5 cc. Jan. 31, fine tremors. Feb. 1, complete paralysis, both arms; killed.

Medulla, Right and Left, Pooled.—Monkey F84-0, inoculated Nov. 16, 1932; 1.5 cc. Nov. 25, partial paralysis both legs. Nov. 28, complete paralysis; killed.

Cerebellum, Right and Left, Pooled.—Monkey F85-7, inoculated Nov. 29, 1932; 1.5 cc. Remained well.

Spinal Cord, Cervical.—Monkey F85-5, inoculated Nov. 29, 1932; 1.5 cc. Remained well.

Spinal Cord, Lumbar.—Monkey F85-6, inoculated Nov. 29, 1932; 1.5 cc. Remained well.

Dorsal Root Ganglia, Cervical and Lumbar, Right and Left, Pooled.—Monkey F85-4, inoculated Nov. 29, 1932; 1.5 cc. Remained well.

4. Invasion of the spinal cord and ganglia. Tremor, hyperesthesia. 7th day (approximately).

In direct succession to the fourth stage, is the stage of flaccid paralysis (8th day, approximately) in which the function of the anterior horn cells ceases as they are destroyed. The final stage of healing, common in man, is rarely seen in the monkey.

CONCLUSIONS

1. About 4 days after intranasal instillation, the virus of poliomyelitis establishes its initial focus, within the central nervous system, in the olfactory bulbs. It apparently reaches this structure through the axons of the olfactory nerves after primarily infecting the olfactory cells of the nasal mucosa.

2. From this initial focus, the virus spreads (on the 5th and 6th days) through the olfactory tracts and their connections in the brain stem. A secondary focus in the hypothalamus is first established. From this, two main channels can be discerned: first, to the medulla; second, to the thalamus and midbrain.

3. On the 7th day, virus can first be detected in the spinal cord. It is widespread but is found in larger amounts in the cervical than in the lumbar segments. It is present in both the anterior and posterior horns, either in equal amounts or in slightly larger amounts in the posterior. It is also present in the intervertebral ganglia. The surmise is presented that the main route of infection of the cord is not from the medulla (which had been infected as early as the 5th day) but along the sensory tracts, presumably from the thalamus (spinothalamic tracts).

4. Certain portions of the central nervous system were never found to contain demonstrable quantities of virus: these were the cortex of the frontal and parietal lobes (neopallium), and the cerebellum. The olfactory (archipallial) cortex (hippocampus) was only once found to contain virus; this occurred on the 7th day and in small amounts, and presumably had its source in the olfactory bulbs.

5. The experiments of the 7th day suggest that virus had died out in areas previously infected (in the hypothalamus and thalamus, particularly), while continuing, apparently undiminished, in the midbrain and medulla, and spreading to the cord. These observations are in

Subinoculations

Left Olfactory Bulb.—Monkey F86-4, inoculated Dec. 14, 1932; 1.5 cc. Dec. 20, poliomyelitis. Dec. 23, complete paralysis; killed.

Right Olfactory Bulb.—Monkey F89-5, inoculated Jan. 5, 1933; 2.0 cc. Jan. 12, poliomyelitis. Jan. 13, paralysis both arms. Jan. 14, complete paralysis.

Hippocampus, Right and Left, Pooled.—Monkey F89-6, inoculated Jan. 5, 1933; 2.0 cc. Jan. 17, poliomyelitis. Jan. 18, paralysis both arms. Jan. 19, complete paralysis; killed.

Cortex; Right and Left Sides Pooled; Precentral and Postcentral Gyri; Anterior Pole of Frontal Lobe.—Monkey F89-7, inoculated Jan. 5, 1933; 2.0 cc. Remained well.

Left Hypothalamus.—Monkey F86-5, inoculated Dec. 14, 1932; 1.5 cc. Remained well. Susceptibility shown by poliomyelitis on 9th day after intranasal inoculation, Jan. 10, 1933.

Right Hypothalamus.—Monkey F93-0, inoculated Jan. 28, 1933; 1.5 cc. Remained well.

Left Thalamus.—Monkey F86-6, inoculated Dec. 14, 1932; 1.5 cc. Remained well. Susceptibility shown by poliomyelitis on 8th day after intranasal inoculation, Jan. 10, 1933.

Right Thalamus.—Monkey F89-4, inoculated Jan. 5, 1933; 2.0 cc. Jan. 20, paralysis both arms. Jan. 21, complete paralysis.

Midbrain, Right and Left, Pooled.—Monkey F92-7, inoculated Jan. 23, 1933; 1.5 cc. Jan. 31, tremors. Feb. 2, complete paralysis; killed.

Medulla, Right and Left, Pooled.—Monkey F89-3, inoculated Jan. 5, 1933; 2.0 cc. Jan. 11, poliomyelitis. Jan. 12, paralysis both arms. Jan. 13, complete paralysis; killed.

Cerebellum, Right and Left, Pooled.—Monkey F89-8, inoculated Jan. 5, 1933; 2.0 cc. Remained well.

Spinal Cord, Cervical, Anterior Half.—Monkey F92-4, inoculated Jan. 23, 1933; 1.5 cc. Jan. 30, slight nervousness. Jan. 31, tremors, paralysis right arm. Feb. 2, complete paralysis. Feb. 4, died.

Spinal Cord, Cervical, Posterior Half.—Monkey F92-4, inoculated Jan. 23, 1933; 1.5 cc. Jan. 30, nervousness. Jan. 31, tremors, paralysis left leg. Feb. 2, complete paralysis. Feb. 3, died.

Spinal Cord, Lumbar, Anterior Half.—Monkey F92-6, inoculated Jan. 23, 1933. Feb. 2, tremors; paralysis both legs. Feb. 3, complete paralysis; killed.

Spinal Cord, Cervical and Lumbar, Posterior Half.—Monkey F89-9, inoculated Jan. 5, 1933; 2.0 cc. Jan. 12, poliomyelitis. Jan. 13, almost complete paralysis. Jan. 13, killed.

Spinal Cord, Lumbosacral.—Monkey F86-7, inoculated Dec. 14, 1932; 1.5 cc. Remained well. Susceptibility shown by poliomyelitis 8 days after intranasal inoculation, Jan. 10, 1933.

Dorsal Root Ganglia, Left.—Monkey F88-0, inoculated Dec. 14, 1932; 1.5 cc. Dec. 26, poliomyelitis. Dec. 28, complete paralysis; killed.

13. Schultz, E. W., and Banham, F. D., *Am. J. Pub. Health*, 1930, 20, 771.
14. Harbitz, F., and Scheel, O., Pathologisch-anatomische Untersuchungen über akute Poliomyelitis und verwandte Krankheiten von den Epidemien in Norwegen 1903-1906. Videnskabs-Selskabets Skrifter, I. Matematisk-naturvidenskabelig Klasse, 1907, No. 5, Christiania.

TABLE VIII
Summary of Experimental Results

Day after inoculation.....	4			5			6			7		
	Left	Both	Right	Left	Both	Right	Left	Both	Right	Left	Both	Right
Olfactory bulb.....	+++		-	+++		+++	+++		0	+++		+++
Cortex.....												
Hippocampus.....												
Pre-, postcentral gyri.....		0			0			0				
Interbrain.....		0			0			0				
Hypothalamus.....												
Thalamus.....	0											
Midbrain.....			-	++		++	+++		0	0		0 +
Pons-medulla.....		-			0		++		++			
Cerebellum.....		0			0		++		++			
Cord.....		-			++							
Cervical, undivided.....					0							
Cervical, anterior half.....		0(a)										
Cervical, posterior half.....					0							
Lumbar, undivided.....												
Lumbar, anterior half.....		0(a)										
Lumbar, posterior half.....												
Cervical and lumbar, posterior half.....												
Lumbosacral, undivided.....												
Dorsal ganglia.....		0(a)		-			0			+		++

0(a): 3 specimens pooled; single test. ++++: incubation in test animal, 4 to 5 days. +++: incubation in test animal, 6 to 7 days. ++: incubation in test animal, 8 to 9 days. +: incubation in test animal, 10 days or more. -: untested.

13. Schultz, E. W., and Banham, F. D., *Am. J. Pub. Health*, 1930, **20**, 771.
14. Harbitz, F., and Scheel, O., Pathologisch-anatomische Untersuchungen über akute Poliomyelitis und verwandte Krankheiten von den Epidemien in Norwegen 1903-1906. Videnskabs-Selskabets Skrifter, I. Mathematisk-naturvidenskabelig Klasse, 1907, No. 5, Christiania.

the homolateral hypothalamus; in the contralateral bulb; and in a more distant area, the medulla oblongata. It is not found in the olfactory cortex (hippocampus), thalamus, midbrain, cerebellum or spinal cord. Its presence in the right olfactory bulb may have been due either to passage from the left bulb through the anterior commissure or to spread of infection from the nasal mucosa of the left naris to that of the right and so up the right olfactory nerves. Its presence in the homolateral hypothalamus was anticipated since this structure (the mamillary region, especially) is directly connected with the olfactory bulb through one of the olfactory tracts and also indirectly, by way of the hippocampus. It would appear that the virus had followed the outgoing olfactory fibers along their basal pathways, which are the shortest. The absence of virus from the olfactory cortex (hippocampus) in this and all but one of the other animals is extremely interesting, since the greater portion of the outgoing olfactory pathways in all higher vertebrates is long circuited through the cortex before they go in the interbrain and midbrain. To explain the phenomenon, it is necessary to refer to Fairbrother and Hurst's (10) conclusion that the cortex of the brain in general affords an unsuitable ground for implantation and survival of poliomyelitis virus.

That the medulla should show infection before the thalamus or midbrain had not been anticipated and called our attention to the possible existence of intimately connected tracts between the hypothalamus and medulla. Recent studies, elsewhere (2) reviewed, have shown important functional connections between the hypothalamus and medulla concerned largely in the higher control of various sympathetic and parasympathetic functions, and the probability that the corresponding anatomical tract is, in part at least, the formatio reticularis. In histological studies of human poliomyelitis, this structure of ganglion cells and white matter has been found to be quite constantly and heavily involved (Harbitz and Scheel (14)), and the prominence of various sympathetic and parasympathetic symptoms early in the disease may be referable to these lesions in and between the hypothalamus and medulla. The relatively large amount of virus in the medulla, indicated by the appearance of symptoms in the test animal on the 6th day (complete paralysis on the 10th day) indicates further that bulbar tissue (or at least the part in question) is favorable to implantation and multiplication of virus.

Hurst (4) has already pointed out that certain pathological findings in louping ill are similar to those seen in poliomyelitis, and this fact stimulated us to ascertain whether the maladies are immunologically related. Furthermore, the fact that vaccinia protects against small-pox made the problem more intriguing, because of the possibility that we might hit upon a simple method of immunization against poliomyelitis by the use of louping ill virus. With these ideas in mind we undertook the experiments reported at this time.

Methods and Materials

Louping Ill Virus.—The louping ill virus was procured from the Department of Bacteriology, University of Edinburgh, and was designated as follows: "obtained from the Moredun Institute, Animal Diseases Research Association, on 2nd February 1932 in the form of dried brain of sheep 43, 6th May 1931, since February 1932 maintained by intracerebral inoculation in mice." The virus was sent to us at The Rockefeller Institute in an infected mouse brain preserved in 50 per cent glycerol. Shortly after arrival, May 9, 1932, the material was washed several times in sterile Locke's solution and thoroughly ground in a mortar. Sufficient Locke's solution was then added to make a 10 per cent emulsion which was used to inoculate intracerebrally a group of mice and a monkey. After several intracerebral passages in monkeys and mice the brains of animals killed at the height of the disease were placed in 50 per cent glycerol under a vaseline seal and stored at 0°C. This material constituted the source of supply of virus for our experiments.

Poliomyelitis Virus.—The virus of poliomyelitis was supplied to us by Dr. Flexner in the form of a Berkefeld N filtrate of a 5 per cent emulsion prepared from the spinal cord of a monkey infected with the "mixed virus" strain.

Animals.—Only Indian monkeys (*Macacus rhesus*) were used in the experiments. The mice were of the Rockefeller Institute albino strain (*Mus musculus*).

Cultures.—The presence or absence of ordinary bacteria in infected organs was determined by means of aerobic and anaerobic cultures in meat infusion broth, pH 7.8.

Sections.—The clinical diagnosis of louping ill in each monkey was confirmed by means of histological studies of the central nervous system. In mice, however, histopathological examinations were made only when doubt arose as to the true nature of the illness. Tissues were fixed in Zenker's fluid or in a 10 per cent solution of formalin, sectioned, and stained according to Giemsa's method or by means of eosin and methylene blue.

Before undertaking the study of the immunological relation of poliomyelitis to louping ill it was essential for us to obtain monkeys immune to each of the maladies. Inasmuch as monkeys immune to poliomyeli-

spinal cord. At the same time there is evidence that it has died out or disappeared from some of the previously infected areas, thus confirming the experience of Fairbrother and Hurst with intracortical inoculations. It is still present in high concentration in the left and right olfactory bulbs; indicating either that virus has continued to ascend from the olfactory nerves or that the tissues here are favorable to survival, as well as to initial implantation and multiplication, of the virus. The disappearance from the homolateral (left) hypothalamus is particularly interesting, since on the previous day, it had been present here in large amounts.

The contralateral side was negative. Virus had also disappeared from the homolateral (left) thalamus, but was still present, though in minimal amount (15 day incubation) in the contralateral thalamus. Its appearance in small amounts (12 day incubation) in the hippocampus—the only positive result in the entire series from the end brain—is of interest as evidence of the predominant infection of the olfactory tracts, but the lateness and slightness of this cortical involvement suggests that it is probably incidental and secondary.⁴

In the 7th day experiments, an attempt was made to determine what differences, if any, might be detected between the anterior (motor) and posterior (sensory) portion of the cord, as well as between different levels. If the anterior gray matter contained larger amounts of virus, it might be inferred that the virus had descended along the tracts connecting the higher centers in the brain stem directly with the anterior horn cells (rubrospinal, tectospinal, vestibulospinal, reticulospinal tracts, etc.). If, on the other hand, the posterior horns were more heavily infected, it might be inferred that it had descended along the main sensory tracts (spinothalamic tract, columns of Goll and Burdach). Clinical evidence, particularly the character of the preparalytic symptoms in man, points rather clearly to the spinothalamic (2).

The present experiments fail to answer this question unequivocally, perhaps due in part to the necessity of using immediately contiguous

⁴ Harbitz and Scheel (14) have described inflammatory lesions in this area. However, no characteristic symptoms (olfactory hallucinations) derived from such involvement in man have been reported. The point has not been sufficiently studied by clinicians.

the 4 treated and 2 untreated monkeys received intracerebrally 1 cc. each of a 10 per cent virus-brain emulsion. 3 of the treated monkeys developed typical louping ill, 1 on the 8th, 1 on the 10th, and 1 on the 11th day after injection. These animals were only moderately ataxic and gradually recovered. The 4th treated monkey showed symptoms of the disease on the 20th day after the intracerebral inoculation and was killed (it probably would have recovered) 3 days later for histological studies. The 2 control monkeys died of louping ill.

Intranasal Instillation.—Each of 4 monkeys received 3 intranasal instillations, 1 a week, of a 15 per cent virus-brain emulsion; 2 monkeys received 0.5 cc. amounts, the others 3 cc. amounts. None of the monkeys developed symptoms of louping ill. 2 weeks after the last instillation, the 4 treated and 2 untreated monkeys received intracerebrally 1 cc. each of a 10 per cent virus-brain emulsion. All of the animals developed louping ill and died.

It is obvious from a consideration of the experiments described above that the intraperitoneal injection of virus was the only method that completely protected the monkeys. Of 8 monkeys that were inoculated in this manner, none developed louping ill on subsequent intracerebral injection of an amount of virus sufficient to cause the disease in untreated animals. When immunization of the monkeys was attempted by instillation of the virus into the nares, no resistance was developed, and all 4 of the animals, when tested, developed typical louping ill. All of the monkeys that received immunizing injections intramuscularly also developed the disease when they were tested by intracerebral inoculation. 3, however, recovered almost completely and the condition of the 4th was such that death from the disease seemed unlikely when it was killed for pathological examination. In this animal, the incubation period was 20 instead of the usual 8 days. Since these were the only monkeys in our experience that recovered from the disease after signs had developed, it seems justifiable to conclude that they had acquired partial immunity due to the intramuscular injections of virus.

Inoculation of Louping Ill Immune Monkeys with the Virus of Poliomyelitis

As a result of our experiments on the immunization of monkeys against louping ill, animals refractory to that malady were available for inoculation with the virus of poliomyelitis. These monkeys, having been bled to secure serum for the neutralization experiments that will be described later, were handled in the following manner.

tion in continuous series from the nose to the spinal cord, and, since the portal of entry is probably the same in man, it appears reasonable to suppose that the pathways of infection are similar in the human disease. Considerations of the human symptomatology and distribution of pathological lesions elsewhere (2) discussed point to the same conclusion.

All these considerations, experimental, pathological and clinical, appear to justify a conception of poliomyelitis as an infection of nervous tissues alone throughout its course, by a virus that is rather strictly neurotropic. Even within the nervous system itself a sharp tendency to preferential localization is clearly seen. The neopallial portions of the end brain and the cerebellum were never found to contain virus in our experiments, and the archipallial portion of the telencephalon (hippocampus) contained it but once and then late and in relatively small amounts. Moreover, the involvement of the diencephalon (hypothalamus, thalamus), while marked for a time, appeared to be transient. The suitability of the central nervous tissues to implantation of the virus appears to be limited to the olfactory bulbs, brain stem (exclusive of the cerebellum) and cord; and the conditions suitable for survival of the virus, to the olfactory bulbs, midbrain, medulla and spinal cord. The clinical course in the majority of human cases indicates (2) that the anatomical range of optimal conditions for survival of the virus may be much more limited than in the monkey, since permanent damage is usually left only in a portion of the anterior horns of the cord.

During the preparalytic period in the monkey (the same may be assumed to hold true of man), it may be convenient to distinguish four successive phases of poliomyelitic infection.

1. Implantation in the olfactory mucosa. Infection of the olfactory cells, and ascent through the axons of the olfactory nerves. First 3 days (approximately). No signs or symptoms.

2. Invasion and implantation in the olfactory bulb (initial central nervous focus). No signs or symptoms. 4th day (approximately).

3. Descent of virus through the olfactory tracts; establishment of secondary foci in the interbrain (hypothalamus, thalamus), midbrain and medulla. No disturbances except occasionally fever, and later, general nervousness and apprehensiveness. 5th and 6th days (approximately).

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from the animals, the brains were stored for 24 hours in separate containers in an ice box while bits of each were tested by means of cultures for the presence of ordinary bacteria. The brains free from bacteria were pooled and ground in a mortar. Sufficient Locke's solution was then added to make a 20 per cent emulsion which was centrifuged at 2000 R.P.M. for 3 minutes. The supernatant fluid was removed and again centrifuged. Then decimal dilutions of the supernatant fluid from the 2nd centrifugation were made with Locke's solution. Portions of each dilution were mixed with an equal amount of the serum, the neutralizing properties of which were being investigated. The mixtures were allowed to stand for 2 hours at room temperature and then for 1 hour in a refrigerator at 0°C. 0.03 cc. of each of the mixtures were then injected intracerebrally into each of 6 mice. The animals were observed for 18 days and the number of deaths in each group was recorded. No mouse that succumbed sooner than the 4th day after injection was considered to have died of louping ill.

In the manner described, an experiment was made in which serum from a louping ill immune monkey, serum from a normal monkey, and Locke's solution were each mixed with a set of decimal dilutions of louping ill virus. The results are summarized in Table I. Inasmuch as no deaths occurred in any group that received virus diluted more than 10^{-4} , the results with greater dilutions have been omitted from the death incidence calculations. Of the mice receiving virus mixed with normal monkey serum 70 per cent died, while among those that received virus plus louping ill immune serum only 21 per cent succumbed. The average time of death in the former group was 7.1, in the latter 9.4 days. From these results it appears that sera from monkeys that have recovered from louping ill are capable of neutralizing the virus. In the summary of the experiment shown in Table I, however, one finds that the incidence of death, 9 per cent, is peculiarly low in the mice that received virus dilutions that had been allowed to stand in contact with Locke's solution instead of serum. Since it is highly improbable that Locke's solution contains specific neutralizing antibodies, it seemed likely to us that the virus was damaged by a toxic action of the Locke's solution. Accordingly an experiment was undertaken to determine whether this actually occurred.

Decimal dilutions of a virus emulsion were mixed with equal volumes of Locke's solution. 0.03 cc. of each of these mixtures were immediately injected intracerebrally into each of 6 mice. The remaining portions of the mixtures were

harmony with the general contentions of Fairbrother and Hurst that virus is better adapted to survival in the lower portions of the cerebro-spinal axis than in the higher.

6. The conception here presented of the manner of entrance and routes of propagation of the virus of poliomyelitis in the experimental animal appears to be in essential agreement with the clinical and pathological characteristics of the disease in man. Both the experimental disease and the disease as it occurs in man appear to present the features of an infection spread through nervous tissue only. It is unnecessary to assume that at any stage of its progress, during the incubation period or later, systemic or general extraneurvous infection is present.

For a more detailed review of the human pathology and symptomatology, as well as of the anatomical and experimental data relevant to the subject, and for a more exhaustive discussion of the broad conception of poliomyelitis as primarily and essentially a disease of the nervous system, the reader is referred to another paper (2).

We are deeply indebted to Professor Edwin W. Schultz of the Department of Bacteriology at Stanford University who generously placed the facilities of his laboratories at our disposal and so made possible the prosecution of a study which otherwise would have been extremely difficult, if not impossible, for us to carry out.

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that received the mixtures allowed to stand for 3 hours only 30 per cent died (average time of death, 10 days). Such results are similar to those obtained when dilutions of herpetic virus or the virus of yellow fever are allowed to stand in contact with physiological salt solution, and have been attributed to the toxicity of the diluting solution. Consequently, in subsequent neutralization experiments, normal monkey serum instead of Locke's solution was used as a control diluent.

TABLE II
Summary of Experiment to Show the Toxic Action of Locke's Solution on Louping Ill Virus

III Virus

Dilution of virus	No. of mice inoculated	No. of deaths	Percentage of deaths	Day of death	Average time of death
Virus dilutions made with Locke's solution injected immediately					
10 ⁻¹	6	4	65	5, 7, 8, 8	6.7 days
10 ⁻²	5	4	80	5, 6, 6, 7	
10 ⁻³	5	3	60	7, 7, 8	
	16	11	70		
Virus dilutions injected after standing for 3 hrs. in contact with Locke's solution					
10 ⁻¹	5	1	20	7	8.7 days
10 ⁻²	5	1	20	8	
10 ⁻³	6	2	35	6, 14	
	16	4	25		

Having found that sera obtained from monkeys immune to louping ill contain antibodies capable of neutralizing the virus, we proceeded to determine whether such antibodies are present in the sera of monkeys that had recovered from poliomyelitis. 2 experiments in which louping ill immune serum, poliomyelitis immune serum, and normal serum were mixed respectively with decimal dilutions of louping ill virus and handled as described above were performed. The findings have been brought together in Tables III and IV. The results recorded in the former table show that none of the mice that received mixtures of

OBSERVATIONS ON THE IMMUNOLOGICAL RELATION OF POLIOMYELITIS TO LOUPING ILL

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The fact that the etiological agent of louping ill is a filter-passing virus (1) capable of producing in monkeys and mice a disease somewhat similar to poliomyelitis induced us to determine whether any immunological relation exists between the maladies. The results of our investigations are presented in this communication.

Louping ill is a natural disease of sheep in Scotland and the northern part of England. The mass of literature which followed Duncan's original description of the malady in 1807 (2) has been fully reviewed by Pool (3) and indicates the serious nature of the scourge. Only lately, however, has the disease been separated from a number of other maladies giving rise to similar symptoms. The clinical manifestations of louping ill, tremors, hyperesthesia, ataxia, paresis, and paralysis, are caused by a disintegration of nerve cells among which the anterior horn cells of the cord and the Purkinje cells of the cerebellum are particularly involved. In addition to the destruction of neurons, a generalized hemic engorgement of the central nervous system and perivascular round cell infiltrations are usually observed. Louping ill has been transmitted experimentally to monkeys (4, 5), mice (1, 6, 4), swine (7), and probably to rats (1). Rabbits and guinea pigs, however, are apparently not susceptible (1). In general, the experimental disease is similar to the natural infection in sheep. In different hosts the symptoms are not necessarily identical, and such variations are probably occasioned by the type of nerve cell that is particularly involved. For instance, in monkeys, ataxia is the major symptom and is caused by a widespread disintegration of Purkinje cells in the cerebellum, while in mice, tremor, hyperesthesia, incoordination, weakness, and paralysis caused by a destruction of cells in the cerebrum and cord are the outstanding signs. Although no inclusion bodies have been found in the tissues of sheep, pigs, or monkeys, Hurst (4) has observed in the cytoplasm of nerve cells of mice acidophilic bodies which he believes belong to the general class of cytoplasmic inclusions.

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litis serum died (average time of death after inoculation, 6.7 and 7.1 days), while only 22 per cent of the animals that received mixtures of virus and louping ill immune serum succumbed (average time of death after inoculation, 10.2 days). Such results substantiate our earlier

TABLE IV
Summary of an Experiment to Determine whether Sera from Monkeys Recovered from Poliomyelitis Are Capable of Neutralizing Louping Ill Virus

Experiment to Determine whether Sera from Monkeys Recovered from Poliomyelitis Are Capable of Neutralizing Louping Ill Virus					
Dilution of virus	No. of mice inoculated	No. of deaths	Percentage of deaths	Day of death	Average time of death
Normal monkey serum plus virus dilutions					
10 ⁻¹	5	4			6.7 days
10 ⁻²	5	4	80	6, 7, 8, 8	
10 ⁻³	6	6	80	6, 6, 6, 6	
10 ⁻⁴	6	3	100	6, 6, 6, 6, 7, 9	
	22	17	50	6, 7, 8	
			77		
Poliomyelitis immune serum plus virus dilutions					
10 ⁻¹	6	5	85	6, 6, 6, 7, 9	7.1 days
10 ⁻²	6	5	85	6, 6, 7, 8, 9	
10 ⁻³	6	2	35	6, 6	
10 ⁻⁴	5	4	80	7, 7, 8, 9	
	23	16	70		
Louping ill immune serum plus virus dilutions					
10 ⁻¹	6	4	65	9, 10, 11, 11	10.2 days
10 ⁻²	6	0	0		
10 ⁻⁴	6	0	0		
	18	4	22		

findings in regard to the presence of neutralizing antibodies in louping ill immune serum and also clearly indicate that no such antibodies for louping ill virus are demonstrable in the sera of monkeys that have recovered from poliomyelitis.

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were supplied by Dr. Flexner, it only remained for us to devise a safe method of immunizing monkeys against louping ill.

Immunization of Monkeys against Louping Ill

It is common knowledge among shepherds that sheep which survive natural infection of louping ill are immune to the disease. Furthermore, Grieg, Brownlee, Wilson, and Gordon (1) have shown that sheep become resistant to louping ill following subcutaneous or intradermal injection, or intranasal insufflation of the virus. The last two methods are not without danger, however, since a certain number of the sheep develop symptoms of the disease during the process of immunization. Findlay (5) was able to immunize monkeys by intracerebral inoculation of sublethal doses of the infectious agent. The intracerebral method of immunization is obviously unsuitable for use in man. Furthermore, we were desirous of obtaining information regarding the production of disease of the central nervous system when the virus was introduced into the body in several different ways. Consequently, we attempted to immunize monkeys by intraperitoneal and intramuscular injections of virus and by intranasal instillation of the active agent.

Glycerolated infectious monkey brain was thoroughly ground in a mortar with alundum, and sufficient Locke's solution was then added to make a 10-15 per cent emulsion. After centrifugation at about 1000 R.P.M. for 5 minutes, the supernatant liquid was removed and used for the immunizing inoculations. A fresh emulsion was prepared for each set of inoculations and was tested for the presence of active virus by means of intracerebral inoculation of 0.03 cc. of the material into each of 6 mice. 16 monkeys received repeated inoculations of the active brain emulsions, and were then tested for immunity against louping ill. The results obtained are detailed below.

Intraperitoneal Injection.—Each of 8 monkeys received 3 intraperitoneal injections, 1 a week, of 1-5 cc. of a 15 per cent virus-brain emulsion. At no time did the monkeys have fever nor did they show signs of involvement of the central nervous system. 2 weeks after the last inoculation, the 8 treated and 6 untreated monkeys received intracerebrally 1 cc. each of a 10 per cent virus-brain emulsion. None of the immunized animals became sick, while all of the controls developed louping ill and died.

Intramuscular Injection.—Each of 4 monkeys received 3 intramuscular injections, 1 a week, of 3 cc. of a 15 per cent virus-brain emulsion. The animals remained well during the period of immunization. 2 weeks after the last inoculation,

ability to breed animals showing the abnormalities and to follow their development through embryonic and postnatal life affords a new method of approach to such problems.

The present paper describes the deformities as they occur in the rabbit, compares them with those found in man, and summarizes briefly the results of studies on their pathogenesis.

Types of Synostosis

Examination of a large series of skulls of different ages shows that in the rabbit there are four distinct types of deformity based on premature sutural fusion and depending upon the suture or sutures affected. These are illustrated in Figs. 1 to 8 and may be designated as scaphocephaly, trigonocephaly, plagiocephaly, and oxycephaly.

In order to avoid confusion a definition of these terms is necessary. The normal skull of the rabbit is illustrated in Figs. 1 and 6. Scaphocephaly (Figs. 3 and 4) is used in reference to the deformity resulting from fusion of the sagittal suture, plagiocephaly (Fig. 8) to that resulting from fusion of a single segment of the coronal, left or right, trigonocephaly (Fig. 5) to fusion of both coronals, and oxycephaly (Figs. 2 and 7) to fusion of both coronals and the sagittal suture.

These various types are common in certain breeds of rabbits and apparently are in no way incompatible with healthy cage life. Casual observation would not distinguish affected animals from normal members of the same litter, and although close examination may separate a shortened or asymmetrical head, correct differentiation can only be made by careful palpation. In this respect the deformities differ from those occurring in man. In man they produce a striking physiognomy and an unsightliness which prompted Homer's use of oxycephaly in portraying Thersites as the ugliest of men who ever came to Ilium.

Associated Abnormalities

Syndactylism and other physical anomalies occasionally associated with craniosynostosis in man have not been found in the rabbit. It is of interest to note, however, that these cranial variations were first recognized in a line of closely inbred rabbits which showed, among other abnormalities, an extreme adiposity. In two other inbred lines cryptorchism is of frequent occurrence (hereditary). One of these

Each of 4 monkeys immune to louping ill and 2 normal monkeys (controls) received intracerebrally 0.8 cc. of Locke's solution plus 0.2 cc. of a Berkefeld N filtrate of a 5 per cent cord emulsion prepared from a monkey with poliomyelitis induced by the "mixed virus" strain. All of the animals developed poliomyelitis and died or were sacrificed when death was imminent. The louping ill immune monkeys and the control animals responded to the infection in a similar manner.

From the experiment just described, it seems evident that monkeys refractory to louping ill virus introduced intracerebrally are not immune to the virus of poliomyelitis administered in a similar manner.

Inoculation of Poliomyelitis Immune Monkeys with the Virus of Louping Ill

Monkeys that had recovered from attacks of poliomyelitis were bled to secure serum for neutralization experiments and then tested in the following manner for immunity to louping ill.

3 monkeys that had survived attacks of poliomyelitis induced by intracerebral or intracisternal injections of the "mixed virus" strain were supplied by Dr. Flexner. 2 of the animals had had the disease 17 months and 1, 10 months prior to the time they were received by us. The 3 poliomyelitis immune monkeys and 3 normal monkeys (controls) received intracerebrally 1 cc. each of a 10 per cent emulsion prepared from the brain of a monkey with louping ill. All of the animals developed louping ill and died. The monkeys immune to poliomyelitis and the control animals responded to the virus in a similar manner.

The above experiment indicates that monkeys which have recovered from attacks of poliomyelitis are not immune to louping ill virus introduced intracerebrally.

Neutralization Tests

In addition to the experiments already described it seemed advisable for us to pursue the investigation of the immunological relation of poliomyelitis to louping ill by testing the sera of monkeys immune to poliomyelitis for the presence of antibodies capable of inactivating the virus of louping ill. Before these experiments were undertaken, however, it was essential to determine whether such antibodies are present in the sera of louping ill immune monkeys.

The virus emulsions used in the neutralization experiments were prepared from pooled brains of mice killed at the height of a louping ill infection. After removal

parietal with frontal bones. The frontal bones show an anteroposterior shortening, most evident between the bases of their supraorbital processes and the fused coronal sutures. The posterior wings of these processes present an increased and irregular lateral curvature with their thick extremities in contact with the anterior tips of pronounced temporal lines. Frontal eminences are absent and the slope of the bones unchanged from the frontonasal suture to the obliterated coronals. Parietal bones are markedly flattened, sometimes slightly concave, and form more posterior walls than roofs of the cranial cavity. Bosses are not present.

Trigonocephaly.—In trigonocephaly (Fig. 5) the bregmatic peak is absent and a transverse ridge marks the fused coronals. Frontal bones are similar to those described above and at their union with parietals the slope of the calvarium is reversed. Parietals articulate with each other at a slight angle to form a sagittal suture of normal appearance. The degree of parietal flattening makes two classes of this type of skull. In one class these bones are markedly flattened from birth and show small ill defined bosses lying close to the fused coronals. In the other class, the bones are more rounded with well marked bosses in their normal positions.

Plagiocephaly.—In plagiocephaly (Fig. 8) the disproportionate growth between the fused and normal sides causes a complete loss of symmetry with distortion of individual bones. The fused side presents all the characteristics of trigonocephaly, and here also the two different classes may be distinguished. The parietal and frontal bones are shorter and the nasal bones longer in anteroposterior extent on the affected side. The sagittal and frontal sutures, instead of forming a straight line between lambda and nasion, describe a curve convex toward the normal side. The nasal suture presents a slight curve in the reverse direction so that the combined midline sutures of the skull tend to resemble the letter S. The contour of the interparietal, a small oval bone lying between the two parietals and the supraoccipital, is markedly changed, the portion on the affected side being much larger than that on the normal side. The position of the bone is also altered, its plane facing posterolaterally rather than directly posterior as in the normal skull. Fusion of the sagittal suture may occur with this variation, increasing the distortion and accentuating the lateral curvature of the skull.

allowed to stand 2 hours at room temperature and 1 hour in a refrigerator at 0°C. After the period of standing, 0.03 cc. of the different mixtures were injected into each of 6 mice. The results appear in Table II.

TABLE I

Summary of Experiment to Determine whether the Sera from Monkeys Immunized against Louping Ill Contain Neutralizing Antibodies

Dilution of virus	No. of mice inoculated	No. of deaths	Percentage of deaths	Day of death	Average time of death
Louping ill immune serum plus virus dilutions					
10 ⁻¹	6	3	50	8, 8, 10	
10 ⁻²	6	0	0		
10 ⁻³	6	1	15	11	
10 ⁻⁴	6	1	15	10	
10 ⁻⁵	6	0	0		
	24	5	21		9.4 days
Normal monkey serum plus virus dilutions					
10 ⁻¹	6	6	100	6, 6, 6, 6, 7, 7	
10 ⁻²	6	5	80	7, 7, 7, 7, 7	
10 ⁻³	6	5	80	7, 7, 7, 7, 11	
10 ⁻⁴	5	0	0		
10 ⁻⁵	5	0	0		
	23	16	70		7.1 days
Locke's solution plus virus dilutions					
10 ⁻¹	6	2	35	7, 14	
10 ⁻²	6	0	0		
10 ⁻³	5	0	0		
10 ⁻⁴	6	0	0		
10 ⁻⁵	6	0	0		
	23	2	9		10.5 days

Figures in italics have been omitted from calculations.

The results of the experiment summarized in Table II show that of the mice that received the fresh mixtures of virus and Locke's solution 70 per cent died (average time of death, 6.7 days), while of the mice

parietal with frontal bones. The frontal bones show an anteroposterior shortening, most evident between the bases of their supraorbital processes and the fused coronal sutures. The posterior wings of these processes present an increased and irregular lateral curvature with their thick extremities in contact with the anterior tips of pronounced temporal lines. Frontal eminences are absent and the slope of the bones unchanged from the frontonasal suture to the obliterated coronals. Parietal bones are markedly flattened, sometimes slightly concave, and form more posterior walls than roofs of the cranial cavity. Bosses are not present.

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louping ill virus and louping ill immune serum died, while there was a mortality of 53 and 55 per cent respectively among the animals receiving mixtures of virus and normal serum, and virus and poliomyelitis

TABLE III

Summary of an Experiment to Determine whether Sera from Monkeys Recovered from Poliomyelitis Are Capable of Neutralizing Louping Ill Virus

Dilution of virus	No. of mice inoculated	No. of deaths	Percentage of deaths	Day of death	Average time of death
Normal monkey serum plus virus dilutions					
10^{-1}	5	5	100	6, 7, 7, 7, 7	
10^{-2}	6	2	35	7, 9	
10^{-3}	6	2	35	7, 10	
10^{-4}	6	0	0		
10^{-5}	6	0	0		
	17	9	53		7.4 days
Poliomyelitis immune serum plus virus dilutions					
10^{-1}	6	4	70	7, 7, 7, 8	
10^{-2}	6	4	70	7, 7, 8, 10	
10^{-3}	6	2	35	9, 14	
10^{-4}	6	0	0		
10^{-5}	6	0	0		
	18	10	55		8.4 days
Louping ill immune serum plus virus dilutions					
10^{-1}	6	0	0		
10^{-2}	5	0	0		
10^{-3}	6	0	0		
10^{-4}	6	0	0		
10^{-5}	6	0	0		
	17	0	0		

Figures in italics have been omitted from calculations.

immune serum. The findings displayed in Table IV are similar to these just enumerated: 77 and 70 per cent respectively of the mice that received mixtures of virus and normal serum, and virus and poliomye-

Changes in the sella turcica are not marked. The hypophyseal fossa shows no constant change although in oxycephaly and trigonocephaly the posterior clinoid processes are slightly longer and directed more laterally than normal.

Base of the Skull

The base of the skull in oxycephaly and trigonocephaly shows a varying degree of lordosis. In plagiocephaly, the occipital bone together with the tympanic bulbae is twisted toward the fused side. The portion anterior to the spheno-occipital synchondrosis shows a twist in the opposite direction. This distortion, by changing the plane of the cutting edge of the upper incisors, causes unequal wear and there results a gradual shortening of the teeth toward the fused side. Scaphocephalic skulls show no basilar changes. In none of these conditions do the basal foramina show either consistent enlargement or constriction.

A comparison of the different craniosynostotic types shows that the alterations resulting from coronal fusion are more deforming and probably of much greater functional importance than those resulting from sagittal fusion. This observation may have a practical bearing on the treatment of oxycephaly. In man, an operation has been advocated in which artificial coronal and sagittal sutures are made by the excision of strips of cranium, and if performed during early infancy, is said to prevent the usual deformity. In view of the above observation, it seems probable that a similar result would be obtained by limiting the operation to coronal excision alone and foregoing the more dangerous sagittal excision.

SUMMARY

The results of the work presented in the present paper show that louping ill and poliomyelitis immunologically are not closely related. Although relatively few experiments were performed, the data obtained were sufficiently decisive for our purposes. Certainly nothing was found to indicate that one might be able to immunize human beings against poliomyelitis by the use of louping ill virus. In addition to the negative findings, a certain amount of useful information was also secured, namely, (1) monkeys can be solidly immunized against louping ill by intraperitoneal injections of virus and partially protected by intramuscular administrations of the active agent, (2) during the process of immunization no signs of involvement of the central nervous system are manifested, and (3) sera from monkeys immunized in the manner described contain antibodies capable of neutralizing the virus.

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Changes in the sella turcica are not marked. The hypophyseal fossa shows no constant change although in oxycephaly and trigonocephaly the posterior clinoid processes are slightly longer and directed more laterally than normal.

Base of the Skull

The base of the skull in oxycephaly and trigonocephaly shows a varying degree of lordosis. In plagiocephaly, the occipital bone together with the tympanic bulbae is twisted toward the fused side. The portion anterior to the spheno-occipital synchondrosis shows a twist in the opposite direction. This distortion, by changing the plane of the cutting edge of the upper incisors, causes unequal wear and there results a gradual shortening of the teeth toward the fused side. Scaphocephalic skulls show no basilar changes. In none of these conditions do the basal foramina show either consistent enlargement or constriction.

A comparison of the different craniosynostotic types shows that the alterations resulting from coronal fusion are more deforming and probably of much greater functional importance than those resulting from sagittal fusion. This observation may have a practical bearing on the treatment of oxycephaly. In man, an operation has been advocated in which artificial coronal and sagittal sutures are made by the excision of strips of cranium, and if performed during early infancy, is said to prevent the usual deformity. In view of the above observation, it seems probable that a similar result would be obtained by limiting the operation to coronal excision alone and foregoing the more dangerous sagittal excision.

Changes in Other Organs

The brain in these variations is apparently not altered in size but is modified in shape to conform to the altered cranial cavity. The hypophysis presents no gross anatomical change and microscopic examination has not been made. Meninges show no indication of local or general inflammation. Hydrocephaly in any form has not been found associated with oxycephaly or trigonocephaly. Internal hydrocephaly is occasionally found in plagiocephaly in which condition the otherwise normal side is bombos and shows numerous scattered

OXYCEPHALY AND ALLIED CONDITIONS IN MAN AND IN THE RABBIT

By HARRY S. N. GREENE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATES 60 AND 61

(Received for publication, March 10, 1933)

Despite the numerous studies which have been made on oxycephaly and allied abnormalities of the skull, there is still some uncertainty concerning the pathogenesis of these conditions. Some investigators attribute the deformities to primary abnormalities of the sphenoid bone, but the majority opinion is that premature sutural fusion in the calvarium is fundamental in their production.

Injuries in early life, diseases of the cranial bones, abnormalities of the brain, and inflammation of the meninges have been considered as possible causes of the premature bony union, and tuberculosis and syphilis have been emphasized in the theories supporting an inflammatory origin. At present it is, however, quite generally agreed that the sutural fusion arises either as a result of injuries during early embryonic life or actual changes in the germ plasm. Faber (1) suggests that injuries resulting from increased amnionic pressure on the extremely vulnerable differentiating blastema may play an important part. The theory advanced by Rieping (2) is of particular interest. He describes an oxycephalic skull showing ossification centers common for both frontals and parietals situated in the fused coronal sutures, and considers the deformity due to this abnormality in position.

Study of the literature concerning the inheritance of these conditions shows their occurrence in more than one generation of a family to be relatively uncommon, and although more frequent in members of the same generation, the majority of cases are found as isolated instances.

These conceptions are based entirely on studies of human material which is not subject to experimental test or control. A comparable series of synostotic variations has been found in the rabbit (3). The

The question arises, relative to coronal fusion, as to whether or not a true suture separating frontal and parietal bones is ever formed in synostotic skulls. From the evidence at hand, it seems probable that the suture is closed from the start, the displaced frontal center causing frontal and parietal union with earliest bone formation. A similar question applies to sagittal fusion with parietal bosses close to but not in the suture line. In scaphocephalic skulls, it has often been observed that fusion is limited to the sutural region between the bosses, the remainder of the suture, although devoid of denticulations, is unfused. The inference is, therefore, plausible that the closely approximated position of parietal bosses is in some way responsible for ossification in the suture line. A detailed study of embryonic development is necessary before satisfactory answers to the problems can be found, and such a study is now under way.

Inheritance of Craniosynostosis

Breeding experiments show that the craniosynostotic abnormalities which have been described are hereditary variations which may be transmitted unaltered from parents to offspring. It is definitely known that all these types are recessive to the normal but the exact number or combination of genetic factors that produces them is still uncertain. Every breed of rabbit so far tested has been found to carry factors concerned in their production. The widespread occurrence of these factors is illustrated by the appearance of a trigonocephalic animal in a litter resulting from the mating of a trigonocephalic buck with a doe imported from China and showing no visible or palpable skull deformity. The inability to find normal animals from which to obtain true F_1 progeny makes the study of inheritance of these variations extremely difficult. The usual methods of approach are blocked, and the only available means of study consists in a comparison of litters obtained from the same parent by different matings. This is necessarily long and complicated, and no attempt will be made in the present paper to analyze the results so far obtained.

The problem is further complicated by the occurrence of anatomically unrelated cranial variations in litters from craniosynostotic parents. Trigonocephalic and plagiocephalic rabbits do not always breed true and their litters, although largely made up of offspring showing the

lines transmits hydrocephalus and the other a "cretinoid" condition of uncertain etiology. In view of the cases reported in man, in which cranial abnormalities were associated with hemorrhagic icterus, it is also of interest to note that in a litter from the mating of an F_1 doe to her trigonocephalic parent, there were two typical cases of hemorrhagic icterus. Other instances of this condition may have escaped notice. However, none of these conditions can be regarded as a necessary accompaniment of the cranial abnormality. The frequency of association is apparently accidental as synostotic variations have been found in other lines of animals which present none of these irregularities.

Signs of craniosynostosis, other than those present in the skull, are inconstant. A mild degree of exophthalmos is common in one adipose albino line, but no more pronounced in craniosynostotic than in normal members. Examination of the bony orbit shows no structural cause for this condition and its probable explanation lies in increased size of fatty pads. In other lines affected individuals show no exophthalmos, their eyes appearing rather more deeply placed than normal. In plagiocephaly it is usual to find that the eye of the normal side protrudes more than that of the synostosed side. Nystagmus is uncommon although in plagiocephaly a degree of squint occurs compensating for the asymmetrical orbits. Retinal examinations have not been made, but observation of the behavior of many animals gives the impression of deficient sight. In man, cases of coronal fusion ordinarily show considerable exophthalmos which is explained by the shallowness of bony orbits. The failure of this sign to develop in rabbits is accounted for by the absence of such an anatomical alteration.

The Adult Skull

Adult craniosynostotic skulls present structural alterations not generally pronounced at birth and will be described in detail. The normal contour of the calvarium is modified and the size, shape, and relations of individual bones are altered.

The Calvarium

Oxycephaly.—In oxycephaly (Figs. 2 and 7) a raised peak occupies the bregmatic region and sharp ridges indicate the sagittal and coronal sutures obliterated by the angular union of parietal bones and of

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EXPLANATION OF PLATES

PLATE 60

FIG. 1. Lateral view of normal skull of the rabbit.

FIG. 2. Oxycephaly. Note the altered shape of the orbital foramen and contour of the whole orbit as compared with Fig. 1.

FIG. 3. Scaphocephaly. The anteroposterior arch shown by this skull is more marked than usually found in scaphocephaly and is due to conditions other than the sagittal fusion.

PLATE 61

FIG. 4. Calvarium in scaphocephaly showing absence of normal denticulations in the sagittal suture and fusion of the portion lying between parietal bosses (compare with Figs. 5, 6, 8).

FIG. 5. Calvarium in trigonocephaly. Fusion is limited to the mid-section of each coronal segment and these sutures are devoid of normal denticulations. Parietal flattening, frontal shortening, and the changed relationship between these bones is brought out by comparison with the normal skull shown in Fig. 6.

FIG. 6. Calvarium in normal skull.

FIG. 7. Calvarium in oxycephaly. The abnormalities found in Figs. 4 and 5 are combined.

FIG. 8. Calvarium in plagiocephaly showing fusion of the right coronal segment. The abnormalities characteristic of trigonocephaly are seen on the fused side and the deformity resulting from disproportionate growth is obvious. The asymmetrical interparietal bone is seen lying between the two parietals and the occipital bone.

Scaphocephaly.—Fusion of the sagittal suture in scaphocephaly (Figs. 3 and 4) produces a ridged condition of the posterior calvarium in which flattened parietals unite at a considerable angle and the vertical distance between squamosal and sagittal sutures is increased. Parietal bosses are small but distinctly outlined and lie close to the fused sagittal suture. The coronal sutures and the anterior calvarium are not affected.

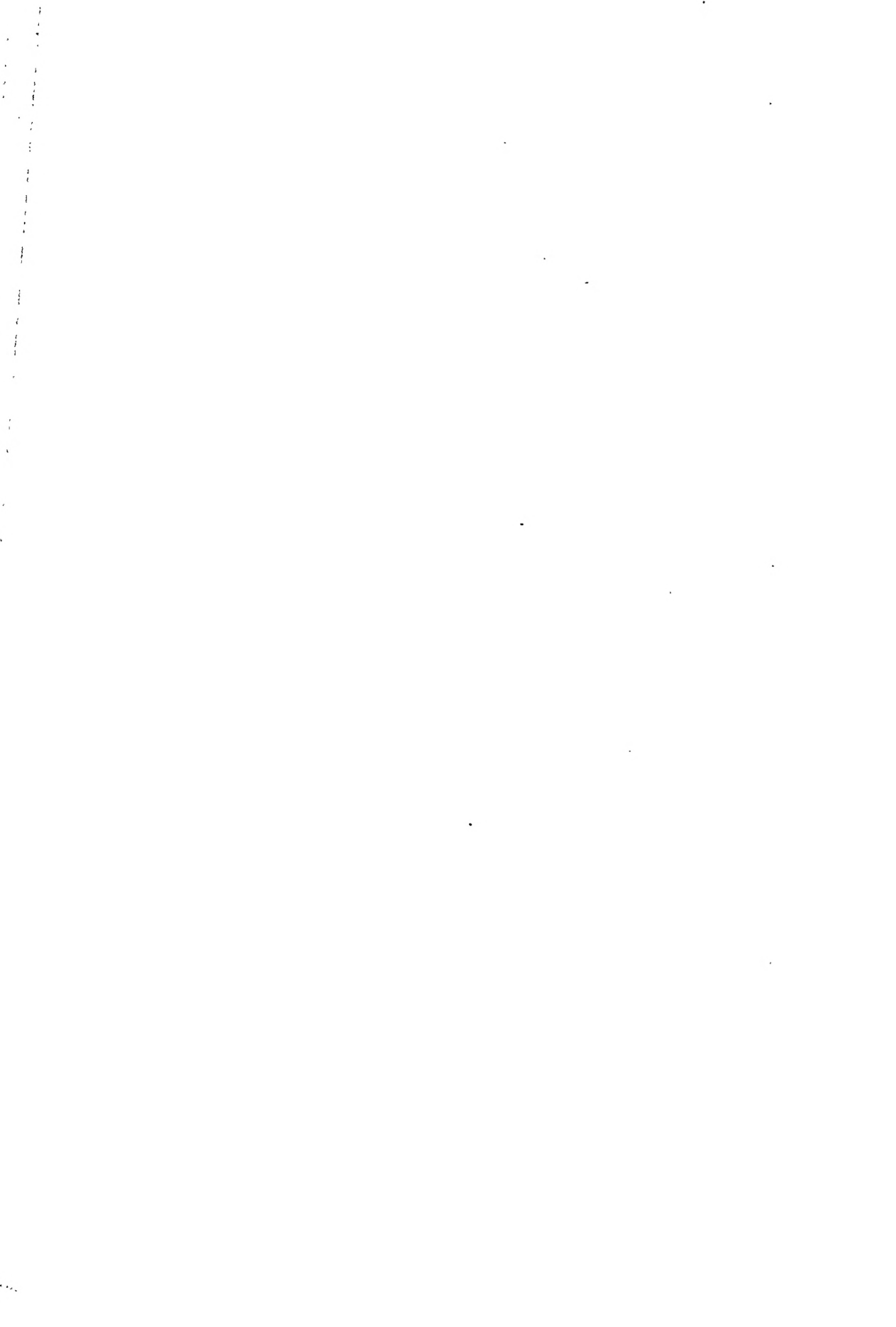
In all these deformities, sometimes called the peak, dome, unilateral dome, and ridge because of the calvarial modification, the affected suture may be obliterated throughout its extent and entirely replaced by bone. In most instances, however, true bony union occurs only in its middle portion, extremities being open although devoid of normal denticulations. Cross-section through a fused suture shows both inner and outer tables thickened and separated by an increased amount of diploe.

The Orbit

Orbital changes are present in oxycephaly, trigonocephaly, and on the fused side in plagiocephaly. In all these variations the circumference of the orbit is increased by greater curvature of the zygomatic arch. Its depth is not decreased, the more perpendicular plane of the frontal portion of the roof being compensated for by its greater extent. The posterior wall shows a more convex curvature both toward the orbit and the ventral surface of the skull, and the alisphenoid occupies a nearly transverse plane. The orbital foramina are not constantly altered but are often smaller than normal or constricted in one diameter.

Cranial Fossae

The cranial fossae are also altered in these deformities. In oxycephaly, the floors of the middle and posterior fossae are deepened and widened and their walls show convolutional markings and deep sinus grooves. The middle fossa is somewhat constricted by bulging of the posterior orbital walls. Similar but less marked changes occur in trigonocephaly, and the disproportions expected from the calvarial asymmetry are evident in plagiocephaly. Middle and posterior fossae are deeper and narrower in scaphocephaly than in the other deformities.



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areas of deficient calcification while the fused side shows nothing but its characteristic deformity. Although not commonly found in scaphocephalic animals, hydrocephaly is unusually frequent in the non-craniosynostotic litter mates of one line. Examination of other organs shows no consistent pathological changes and evidences of syphilis and tuberculosis are entirely absent.

Pathogenesis of Cranial Deformities

Examination of a series of craniosynostotic skulls beginning with the embryonic and continuing through succeeding age periods to the mature adult shows an increasing degree of deformity. Fusion of sutures has been found in feti of the 3rd week of embryonic development, and in such skulls the structural alteration characteristic of the adult is absent. The deformity is thus dependent upon skull expansion and results from inhibition of growth at fused sutures and compensatory increased growth in normal regions. It is noteworthy, in view of the theory that these deformities occur as a result of abnormalities in the sphenoid bone, that sutural fusion is present while the base of the skull is still cartilaginous and the sphenoid entirely normal for that period. Sutural fusion is thus a cause rather than a consequence of the deformity.

It is seen from the foregoing descriptions that certain changes in neighboring bones are constantly associated with sutural fusion. In coronal fusion, with frontal eminences absent, similar structures are found in the fused line and the normal, slightly rounded contour of the frontal bones is lost. In oxycephaly and scaphocephaly, parietal bosses are displaced from their normal positions to lie close to the fused sagittal and the parietal bones are flattened. Also, in a variety of trigonocephaly, parietal bosses are found close to the fused coronals and parietals show a more marked flattening than in the ordinary deformity. It is, therefore, characteristic of these skulls that primary ossification centers are displaced into or close to the sutural line. Accompanying this displacement, is an alteration in bone development resulting in modified contour. It is interesting that when these animals are subjected to an inadequate diet, areas of deficient calcification are found in the regions occupied in non-craniosynostotic rabbits by primary ossification centers.

been demonstrated by the earlier studies of Noetzel (4), by W and Johnstone (5), and by the writer (2). It follows from this that bacteria are employed as inflammatory irritants some information may be obtained concerning the invasive capacities of different microorganisms. This would depend on the type of inflammatory reaction which a given microorganism induces in the host. Pathologists are familiar with the localizing tendency of staphylococci in contrast to the invasive property of the streptococci. Can the behaviors of various pyogenic organisms in tissues be related, in part at least, to their respective properties of creating an early inflammatory reaction at the site of their inoculation which would tend to prevent, by the mechanism mentioned above, their dissemination to the tributary lymphatic nodes? When a vital dye is introduced into an area at various intervals of time after bacterial inoculation of this site, the degree of walling off of the infected focus may be determined by the extent to which the dye can drain into the regional lymphatics.

The observations which are to follow represent a comparison of three pyogenic cocci, *Staphylococcus aureus*, *Streptococcus hemolyticus*, and *Pneumococcus* Type I, with regard to the rapidity of fixation of a vital dye at inflammatory foci set up by these respective organisms. The significance of these data in reference to an understanding of the differences in the invasive properties of these three microorganisms is indicated.

The Retention of Trypan Blue in Areas of Inflammation Induced by Staphylococci

1 or 1.5 cc. of a 1 day old suspension of *Staphylococcus aureus* grown in infusion hormone broth was injected into the dermal tissue of the extensor surface of the foreleg of a rabbit 2 or 3 cm. from the shoulder joint. Either immediately following this injection or after varying intervals of time, 2.5 cc. of a 1 per cent solution of trypan blue in saline was injected into the inflamed area. The same amount of dye was introduced into the other foreleg to serve as a control. All the injections were performed with fine hypodermic needles (gauge 24 or 25). The lymph from the efferent vessel draining the axillary lymphatic node was collected as described in a previous paper (2). Both the lymph and the lymphatic nodes were studied for the presence of the dye.

The results are shown in Table I. When trypan blue was injected in both forelegs of the animal immediately after the inoculation of the

areas of deficient calcification while the fused side shows nothing but its characteristic deformity. Although not commonly found in scaphocephalic animals, hydrocephaly is unusually frequent in the non-craniosynostotic litter mates of one line. Examination of other organs shows no consistent pathological changes and evidences of syphilis and tuberculosis are entirely absent.

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being found to any appreciable extent either in the lymph of the efferent lymphatic vessel or in its lymph node. As this state of affairs was not observed in the axilla of the normal foreleg it is quite likely that with the blockage of lymphatics in the inflamed area a certain amount of dye was carried into the adjacent tissue along with the spread of the edema fluid. A similar diffusion in the perilymphatic tissues had previously been noted in the substernal region when various foreign substances had been injected into an inflamed peritoneal cavity in which the tributary retrosternal lymphatic vessels had been occluded.

Histological studies were made of inflamed areas of about 4 hours duration at a time when trypan blue had been shown to be fixed at the site of staphylococcus inflammation. There was generally a moderate amount of edema as shown by the separation of tissues. Lymphatics were found with their lumina completely or in part occluded by a very delicate fibrinous reticulum (see Fig. 1, Rabbit 7-18). Small deposits of fibrin were also seen in a few areas of such sections. Clumps of staphylococci were frequently observed in close association with fibrinous strands. There was a variable degree of cellular infiltration consisting primarily of polymorphonuclear leucocytes.

Sections of inflamed skin of 19 to about 23 hours duration revealed tissue thickened by a very extensive infiltration of leucocytes predominantly polymorphonuclear in type. At the periphery of the infiltrated zone a reticulum of fibrin varying in its extent was often seen. Lymphatics were found completely occluded by a relatively coarse fibrinous network (Fig. 5, Rabbit 10-24). Cocci were seen in abundance throughout the section.

The axillary lymph node displayed considerable extravasation of red cells. These erythrocytes frequently assumed a peculiar tendency to adhere to the periphery of mononuclear phagocytic cells. Polymorphonuclear leucocytes were found interspersed throughout. In sections of nodes draining an inflamed area of only 6 hours duration numerous cocci were seen mostly within mononuclear cells though in some cases the organisms were located extracellularly.

The results obtained when *Staphylococcus aureus* is used as an inflammatory irritant show that the inability of trypan blue to reach

parental deformity, also contain animals with normal skulls, accessory bones, and reversed sutures. Accessory bones are supernumerary bones in the bregmatic region produced by an accessory suture extending from the coronal to the sagittal suture. They have also been found to occur without relation to craniosynostosis, and in such condition their production seems to depend upon the action of a single pair of hereditary factors. Reversed suture is a condition in which the normal course of the coronal is altered at some distance from the bregma by a more or less abrupt posteromedial change in direction that continues to the sagittal which is thus intersected in its middle third. This condition is relatively rare and little is known of its genetics. It is possible that these variations together with the normal skulls found in such litters are produced by heterozygous combinations of the factors responsible for craniosynostosis. A sufficient number of offspring have as yet not been obtained from oxycephalic or scaphocephalic parents to show that true breeding occurs, but the existence of a genetic as well as a formal relationship between the various types is proved by the fact that all have been obtained by different matings from the same oxycephalic parent.

CONCLUSIONS

Craniosynostosis and the pathological complex with which it is associated in the rabbit are not identical with the abnormalities seen in man; but apparently the two sets of conditions have enough in common to warrant the conclusion that they probably have a common mode of origin. The evidence so far obtained shows that in the rabbit these conditions are hereditary. The factors for the production of the cranial abnormalities are widespread, but the occurrence of deformities is comparatively rare in a general animal population. Inbreeding and selective matings, however, tend to increase their frequency and also the frequency of other abnormalities with which they may be associated, either by chance or by close genetic relationship in the germ plasm. In this way, a pathological complex is built up and perpetuated.

In so far as the cranial abnormalities are concerned, it seems now that oxycephaly and related conditions are variations resulting from a displacement or division of primary ossification centers controlled by hereditary factors.

likely destroys the homologous bacteria *in situ*; perhaps in part by the production of immune bodies. However, this destruction, as pointed out above, is not referable to the reaction of fixation occurring relatively early in the development of an acute inflammatory reaction in tissues unprepared by preliminary vaccination. It is furthermore clear that the experiments of Pacheco are not comparable to the studies of Opie (7) on the fixation of antigen in the Arthus phenomenon, for in the sensitized rabbit the homologous antigen could be injected in an area of perfectly normal skin. The contact of antigen with antibody in the tissue of the sensitized animal gave rise to an acute inflammatory reaction. The development of this inflammatory reaction evidently retarded to a large extent the penetration of the antigen into the circulating blood stream.

The Retention of Trypan Blue in Areas of Inflammation Induced by Pneumococci

In the following series of experiments, varying amounts ranging from 0.1-1 cc. of a 1 day old culture of *Pneumococcus* Type I grown in rabbit blood broth were inoculated into the skin of the foreleg of a rabbit, as in the case of the staphylococcus experiment. Precisely the same technique was followed for determining the time relationship in regard to the fixation of trypan blue. Goodner (9) has recently described the edematous type of lesion which is obtained following skin inoculation with pneumococci. The extension of the local lesion in the rabbit was due largely to direct inoculation of tissues by pneumococci bathed in a tremendous amount of edema fluid that developed under the skin. The edema fluid failed to clot readily owing to the presence of an anticoagulating factor. In view of the latter it became of interest to determine whether the lymphatic vessels are sufficiently affected to prevent the dissemination of a vital dye. In general a similar type of edematous reaction, though perhaps not quite as pronounced as that on the abdomen, described by Goodner, was obtained by injecting a suspension of pneumococci in the skin of the foreleg of a rabbit.

The results as concerns the retention of trypan blue at the site of inoculation with pneumococci appear in Table II. The dye failed to reach readily the regional lymphatics draining the inflamed area, as shown when the lymph was examined 10 or more hours after the injection of the microorganisms (Rabbit 10-06). Histological examination revealed considerable edema with moderate infiltration of polymorphonuclear leucocytes. No fibrin was seen in the tissue except in the dilated lumina of lymphatics. In these vessels a very delicately woven



1



2



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(Greene: Oxycephaly in man and rabbit)

had died with septicemia. These investigators have claimed on the basis of their experiments that it is the antibody content of the body fluids, not the local inflammatory reaction that is responsible for the prevention of the immediate spread of pneumococci in the immune body. The contention seems to be based on the assumption that the presence or absence of leucocytes is the sole criterion for inflammation. The inflammatory reaction, however, is well known to show other manifestations, chief among which is the increase in the permeability of the capillaries. This is not necessarily demonstrable by microscopic examination. By the accumulation of a vital dye from the circulating blood, the increase in capillary permeability has been shown to take place extremely early in the development of the inflammatory reaction, occurring in some cases as early as 2 minutes after the injection of an irritant (3). The writer has previously shown that foreign proteins injected into the blood stream accumulate at the site of inflammation in part because of the increase in capillary permeability (11). When intracutaneous injection of Type I pneumococci is followed several hours later by an intravenous injection of trypan blue, this dye accumulates rapidly at the site of inoculation. It is therefore reasonable to assume in view of these facts that in immune animals circulating antibodies may accumulate very early from the blood stream at the site of inflammation produced by virulent pneumococci.

It has been pointed out previously (3) that the basic mechanism of fixation in inflammation can be reinforced, and this would be especially true in types of specific inflammation, by the addition of secondary factors as agglutinins or precipitins. In this connection it may also be recalled that iron is more effectively fixed than trypan blue in a peritoneal cavity inflamed by a non-specific irritant. This accentuation of fixation is doubtless associated with the precipitation of the ferric salt by the inflammatory exudate. Precipitated iron compounds or particulate matter disseminate less readily than the more soluble trypan blue from the site of inflammation (3). Additional factors, such as precipitation or agglutination, which favor fixation and which may, nevertheless, be important, are considered as secondary factors to the basic mechanism of fixation as demonstrated by the writer, because this mechanism occurs in many forms, if not in all types, of severe inflammation irrespective of any previous immunization, of the type of irritant used, or of the material tested



4



5



6



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(Greene: Oxycephaly in man and rabbit)

The Retention of Trypan Blue in Arcas of Inflammation Induced by Streptococci

The experiments on the fixation of trypan blue were repeated, using a suspension of *Streptococcus hemolyticus* as the inflammatory irritant. The high invasive ability of streptococci is well known. Streptococci, as in the case of *Pneumococcus* Type I, were recovered in some cases by culturing the lymph drawn from the efferent axillary lymphatic vessel a few hours subsequent to the subcutaneous inoculation of the organisms, and they were also found in histologic preparations of the axillary lymph nodes. In Rabbit 7-01 (74 hours inflammation) a section of the liver revealed the presence of the organisms in abundance. In all experiments the Strain S-23 of Lancefield (*Streptococcus hemolyticus*) grown on blood broth was used. Several hours after intracutaneous inoculation of the organisms in the foreleg a reaction appeared in the skin which was usually characterized by varying degrees of redness, necrosis, suppuration, and swelling.

The results as concerns the retention of trypan blue in these inflamed areas are shown in Table III. Fixation of this dye occurred relatively soon after the injection of either staphylococci or pneumococci (1 and 6 hours respectively), but at a considerably later period when streptococci acted as the inflammatory irritant, definite evidence of fixation not being obtained until 45 hours after cutaneous inoculation with the microorganism. An inflamed area caused by streptococci obviously becomes localized considerably later than in the case of staphylococci or pneumococci.

Histological studies reveal patent lymphatics at a time when trypan blue diffuses readily from a streptococcus inflammatory area (see Figs. 3, 6, and 8 and Experiments 6-59, 10-10, and 10-11). However, when the reaction has been going on for 45 hours or longer and fixation of the dye has been demonstrated, one finds in addition to the intense cellular infiltration, lymphatics occluded by dense leucocytic thrombi (see Fig. 4, Experiment 6-42). Areas of delicate fibrinous meshwork

in the same communication to the studies of Field, Drinker, and White (14) who expressed the view that there could be no generalized thrombosis of lymphatics draining an area of extensive sterile inflammation induced by hot water. Evidences recently obtained in this laboratory have demonstrated that in such an inflamed area, as in other types of inflammation previously studied, fixation of trypan blue is primarily due to mechanical obstruction in the form of coagulated plasma in the tissue spaces of the inflamed area, and also in part to the presence of thrombosed lymphatics at the site of inflammation. These observations will form the subject of a separate communication elsewhere (15).

STUDIES ON INFLAMMATION

IX. A FACTOR IN THE MECHANISM OF INVASIVENESS BY PYOGENIC BACTERIA^{*†}

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PLATES 62 AND 63

(Received for publication, February 20, 1933)

In previous studies it has been shown that various foreign materials injected into an acutely inflamed area are fixed *in situ* and fail to disseminate readily to the tributary lymphatic nodes (1). The foreign substances studied included, among others, trypan blue and bacteria (2). The mechanism of the fixation of these substances was shown to be primarily the result of mechanical obstruction in the form both of a fibrinous network and of thrombosed lymphatics at the site of inflammation (3). Fixation has been found to take place in the initial stages of the inflammatory reaction occurring as early as 30 minutes to 1 hour following the injection of an inflammatory irritant. In the majority of experiments chemical irritants were employed; *i.e.*, aleuronat or croton oil.

The intensity and rapidity with which an inflammatory irritant is circumscribed in a tissue area varies with the type of irritant. The speed with which an irritant causes a region to be walled off by mechanical obstruction in the form of either thrombosed lymphatics or of a fibrinous network or of both is necessarily an important index in determining its ability to disseminate ultimately into the circulating blood stream. This holds true if the irritant *per se* can readily drain through lymphatic vessels from the site of inoculation. That bacteria disseminate from the site of inoculation through lymphatics has

* This study was aided by a grant from the DeLamar Mobile Research Fund.

† Read before the American Association of Pathologists and Bacteriologists, Washington, May 9, 1933.

be explained, at least in part, by the type of response induced in the tissues of the host.

In order to determine whether in all of these experiments the volume of culture medium injected with the bacteria played any significant part in the results obtained, two rabbits were injected in the skin of the foreleg with 1 and 1.5 cc. of infusion hormone broth and blood broth respectively. About 17 hours later no signs of inflammation were apparent at the site of inoculation. Trypan blue injected into the area penetrated to the tributary lymphatics as readily as when injected into the normal foreleg. Whatever injury may be produced by the infusion or blood broth is evidently so slight that no fixation of dye occurs. The results obtained are apparently due wholly to the action of the microorganisms inoculated.

In the case of the experiments with streptococci, two of the animals received small amounts of dye in their inflamed forelegs (Rabbits 10-15 and 10-14). This was done in order to determine whether some fixation could be obtained by reducing the amount of dye injected at the site of a streptococcus inflammation. But even small quantities of dye failed to be fixed when introduced 18 or 19 hours following the inoculation of the microorganisms. These observations coupled with the histologic picture of patent lymphatics show that the free diffusibility of trypan blue to the tributary lymphatics in streptococcal inflammation is not the result of an excess of injected dye.

DISCUSSION

The foregoing observations clearly show that the rapidity with which an area of acute inflammation becomes so walled off that foreign substances fail to spread readily from it is a function of the irritant. The intensity and rapidity with which an inflammatory irritant will induce in tissues the formation of a mechanical barrier either in the form of coagulated plasma or of thrombosed lymphatics or both is an important factor in preventing various materials, including the irritant itself, from extending to the tributary lymphatics. Staphylococci, by their high necrotizing power, cause the formation of thrombi in lymphatics and hence fixation within about 1 hour after their introduction into normal cutaneous tissue. The streptococci employed in this experiment, on the other hand, accomplished this only about 45 hours later. The fact may account for the difference in invasive behavior of staphylococcus and streptococcus. It is conceivable, however, that there may also be other factors involved.

The observations reported in this communication present an interesting paradox. Staphylococci tend to remain localized and produce

bacterial suspension, the passage of the dye occurred in both the normal and inflamed side with about equal intensity. If, however, the dye was injected slightly over 1 hour after the bacterial irritant, trypan blue reached the lymph node or its efferent lymphatic vessel in greatly reduced quantities. Evidently the fixation or retention of the dye in a staphylococcus inflamed area occurs very early and is detectable about 1 hour after the onset of inflammation. It is of interest in this connection to note that in a previous study (2) trypan blue has

TABLE I
Retention of Trypan Blue at the Site of Staphylococcus Inflammation

Rabbit No.	Interval between injection of irritant and that of dye	Total duration of inflammation	Presence of dye on inflamed side		Presence of dye on normal side	
			Lymph of efferent lymphatic	Lymph node	Lymph of efferent lymphatic	Lymph node
	hrs.:min.	hrs.:min.				
7-05	0:0	2:23	++	++	++	+++
7-07	0:30	1:52	+++	+++	+++	+++
10-26	1:03	3:33	Faint trace	Trace	+	+
10-26A	1:43	4:00	0	Faint trace	Trace	+
6-61	1:35	4:07	Faint trace	0	++	+
7-18	1:45	4:07		Trace		++
10-27	3:43	5:55	0	0	+	++
10-21	17:38	19:45	0	0	++	++
10-22	17:40	19:55	0	0	++	+
10-20	21:50	23:00	0	Trace		++
10-23	21:05	23:16	0	0	Trace	+++
10-24	20:57	23:28	0	0	++	++

been shown to be fixed as early as 30 minutes after the introduction of aleuronat into tissues.

The cutaneous site of staphylococcus inoculation in the earliest stages showed only moderate swelling and redness. As the inflammatory reaction progressed a typical abscess formed with a discolored necrotic central focus surrounded by a zone of redness. The degree of swelling at this stage (17-23 hours) was as a rule extremely conspicuous. The axillary lymph nodes were moderately enlarged and congested. The dye often diffused from the point of its inoculation through the inflamed tissues almost up to the axilla without, however,

appear to depend on mechanical obstruction in the form of both a fibrinous network and thrombosed lymphatics or thrombosed lymphatics alone at the site of inflammation.

Inasmuch as staphylococci, pneumococci, and streptococci spread from the site of cutaneous inoculation primarily through lymphatic channels, the difference in the rapidity with which mechanical obstruction is set up in the areas inflamed by them will help to explain the differing invasive abilities of these pyogenic organisms.

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EXPLANATION OF PLATES

PLATE 62

FIG. 1. Drawing of a lymphatic lumen in an area of cutaneous inflammation induced by *Staphylococcus aureus* (Rabbit 7-18, Table I). The inflammatory reaction is of about 4 hours duration and trypan blue failed to reach the lymphatic node as readily as under normal circumstances. Note the delicate fibrinous reticulum occluding the lumen. \times about 1400.

FIG. 2. Lymphatic vessel in an area of cutaneous inflammation induced by Pneumococcus Type I. The inflammation is of about 4 hours duration. Trypan blue injected into the area readily drained to the tributary lymphatics (Rabbit 6-63, Table II). The lumen is wholly patent. \times 450.

FIG. 3. Lymphatic vessel in an area of inflammation induced by *Streptococcus hemolyticus*. The reaction is of about 4 hours duration. There is considerable edema. The lymphatic lumen is entirely patent and trypan blue injected into this area drained freely to the regional lymphatics (Rabbit 6-59, Table III). \times 450.

the regional lymphatics occurs at a very early period in the development of the reaction and that this fixation can be satisfactorily explained by the early formation of fibrin in tissue spaces and of thrombi in lymphatics at the site of inflammation.

Recently Pacheco (6) treated an area of skin in guinea pigs with ten daily injections of heat-killed *Staphylococcus aureus*. This led to the proliferation and mobilization of large numbers of macrophages in the area treated. The animals were then left undisturbed for 25 days after which they were inoculated with a living culture of *Staphylococcus aureus* into the skin area. This investigator reported that there ensued an accelerated inflammatory response and healing, with localization of the bacteria in the injected area. In discussing the possible mechanism of this localization Pacheco dismissed the factor of thrombosed lymphatics and of fibrin deposits as the explanation for the effects produced. His experiments, however, have little bearing on the problem of fixation as studied by the present writer. The term "fixation," as used throughout our experimental series, connotes not necessarily the destruction but the inability of substances or bacteria to disseminate readily from the site of acute inflammation to either the tributary lymphatic nodes or to the circulating blood. It is a reaction found in non-specific as well as specific types of inflammation and its intensity seems to depend primarily on the extent of fibrin deposits and on the number of thrombosed lymphatics in the injured area. Only a study in both control and experimental animals of the number of microorganisms recovered in the tributary lymphatic nodes or in the blood stream compared with that found in the inflamed area can give precise information as to the fixation of the microorganisms. Experiments of this type have not been performed by Pacheco. The mere physical appearance of agglomeration or agglutination of bacteria as reported by this investigator in what may perhaps be termed an "immune" area is suggestive but not an adequate demonstration of the fixation of the organisms. It is readily granted, as has been pointed out previously (3) that agglutination or precipitation in any inflammation acting as accessory factors may play a part in accelerating and intensifying fixation, provided, however, that this reaction has been adequately demonstrated to occur. Local tissue immunity as a result of preliminary vaccination very

reticulum could usually be seen under oil immersion magnification which occluded the lumen either wholly or in part. Histological studies of areas inflamed for shorter periods revealed dilated and patent lymphatics with small infiltration of polymorphonuclear leucocytes in tissues distended with edema (Fig. 2, Rabbit 6-63). When the inflammatory reaction is of longer duration (20 hours or over) the dye completely fails to reach the tributary lymphatics. At the same time lymphatic vessels could be found that were completely occluded by dense thrombi (Fig. 7, Rabbit 10-02). In such sections the heavy cellular infiltration was conspicuous but fibrin deposits were strikingly absent from the area of inflammation.

TABLE II
Retention of Trypan Blue at the Site of Pneumococcus Inflammation

Rabbit No.	Interval between injection of irritant and that of dye	Total duration of inflammation	Presence of dye on inflamed side		Presence of dye on normal side	
			Lymph of efferent lymphatic	Lymph node	Lymph of efferent lymphatic	Lymph node
	hrs.:min.	hrs.:min.				
6-63	1:48	4:07	+	++	+	++
10-30	4:17	6:37	Trace	Trace	Trace	++
6-72	6:06	9:37	Trace	+	+	+
10-06	6:22	9:53	Trace	Trace	+	++
10-05	16:38	20:01	Trace	Trace	++	++
10-09	17:38	20:05	0	0	+	++
10-02	20:45	23:10	0	0	+	+
X	25:34	27:25	0	0	+	+

The results listed in Table II indicate that in an area treated with *Pneumococcus* I, fixation of trypan blue occurs relatively early although not as early as in the staphylococcus inflammation. Moreover, this fixation was evidently associated primarily with the blockage of lymphatic vessels at the site of inflammation, inasmuch as a fibrinous meshwork was seldom if ever observed in the tissue spaces.

Rich and McKee (10) have recently removed leucocytes from the circulation by treating immunized animals with benzene. The animals were then reinoculated with virulent pneumococci. The bacteria adhered to each other and to the tissues as they proliferated and were thus held fixed at the site of inoculation for hours after controls

in regard to its disseminating abilities from the site of inflammation. For this reason the rôle of antibodies in the early stages of some types of acute specific inflammatory reaction in possibly intensifying fixation has never been controverted. The question raised here is whether sufficient evidence has been brought forward by Rich and McKee to justify their contention, which, however, may be perfectly true, that the presence of these antibodies at the site of bacterial reinfection is not a part of the inflammatory reaction.

It is likely that in reinfected immune animals agglutination may cause a certain degree of local bacterial destruction, but this, as pointed out in an earlier part of this paper, is referable to other factors than the basic reaction of fixation. It has also been pointed out above that mere evidence of an agglomerative tendency on the part of bacteria in the tissues of an immunized animal, although suggestive, is not a proof of fixation.

It is somewhat difficult in view of the writer's present observation on *Pneumococcus I* to assume that in the experiments of Rich and McKee there was not, as the lesion progressively developed, an additional mechanical obstruction in the form of thrombosed lymphatics which retarded somewhat the passage of the virulent pneumococci. In the immunized animal this retardation was presumably enhanced and accelerated by the accumulation of antibodies in the area where the bacterial irritant produced definite damage to the capillary endothelium.¹

¹ In a recent article which has come out since the present communication was sent to this *Journal*, Rich (12) has reiterated his views that in an immune body pneumococci are primarily held to the tissues through the agency of immune bodies and that local fixation precedes demonstrable microscopic evidence of inflammation. As pointed out above, and also in a previous communication (3), it has been stated that immune bodies, in cases of certain specific inflammation, probably play a part by their concentration at the site of inflammation in the localization and disposal of the irritant. The only question that had previously been under discussion (1) was whether or not an acute inflammation, allergic or otherwise, retards the dissemination of bacteria from its site and thus plays a rôle in immunity. The studies of the writer and of others have answered this question in the affirmative. Several years ago Rich and McCordock (13) expressed strong doubts as to the proof of the ability of the inflammatory reaction to limit the dissemination of bacteria. Rich (12) probably in view of the amount of evidence recently brought forth, has since admitted this fact. This investigator has referred

are also frequently seen. It appears from all these observations that the rapidity with which these three microorganisms establish mechanical blockage of lymphatics draining an inflamed area is greatest in the case of the staphylococci and least in the case of the streptococci. Since it has been shown in previous studies (2, 3) that the presence of

TABLE III
Retention of Trypan Blue at the Site of Streptococcus Inflammation

Rabbit No.	Interval between injection of irritant and that of dye	Total duration of inflammation	Presence of dye on inflamed side		Presence of dye on normal side	
			Lymph of efferent lymphatic	Lymph node	Lymph of efferent lymphatic	Lymph node
	hrs.:min.	hrs.:min.				
6-59	1:44	4:06	+++	++	+++	++
6-71	6:07	10:08	+	+	+	++
10-10	17:05	19:20	+	+	+	+
10-16	17:50	20:14	+	++	++	+ to ++
10-12	17:50	20:40	++	++	++	++
10-15*	18:15	21:30		Faint trace		Trace
10-13	19:15	22:05	++	++	+ to ++	+
10-14†	19:10	22:15	Trace	+	Trace	Faint trace
10-11	23:45	26:15	++ to +++	+++	+++	+++
6-70	30:03	32:39	+	Trace	+	++
10-17A	45:30	47:33	0	0	++	++
10-18	45:25	47:40	0	0	+++	+++
6-58	45:21	47:41	+++	++	+++	+++
10-17	46:19	49:30	0	0	+++	+++
6-69	47:56	50:13	++	++	++	++
6-42	48:16	50:43	0	0	++	+
10-19	70:45	73:35	+	+	++	++
7-01	71:10	74:10	+	+	++	+++

* Smaller quantities of dye injected into each foreleg (amount—1.8 cc. into each foreleg).

† Smaller quantities of dye injected into each foreleg (amount—1 cc. into each foreleg).

coagulated plasma and of thrombosed lymphatics acts as an obstacle to the dissemination of bacteria, it follows that variation in the speed with which bacteria induce the production of such a barrier must be an important factor in explaining differences in their invasive properties. The ability of pyogenic organisms to disseminate can evidently

laboratory animals by injecting the organisms, or the culture filtrates of organisms isolated from patients have signally failed.

Our attack on the problem was based upon the following premises.

(a) Certain of the pyogenic bacteria are capable of producing a toxin which is specifically lethal for leucocytes, particularly for granulocytes (5, 6). This toxin is leucocidin. The production of leucocidin has been reported for *Staphylococcus aureus* (7), *Streptococcus haemolyticus* (5-8), *Streptococcus viridans* (5), and *Pseudomonas aeruginosa* (9), which have been more or less prominently associated with agranulocytosis.

(b) Secondly, one or more of these organisms are almost constantly associated with focal infections (10), which in turn are frequently accompanied by granulocytopenia (11, 12).

(c) Also, a history of focal infection, or lesions with which staphylococci and streptococci are characteristically associated, can be elicited from most of the reported cases of agranulocytosis (*vide* Blanton (13), 1 case; Harkins (14), 8 cases, 100 per cent; Conner *et al.* (15), 11 of 14 cases; also Kastlin (16), Skiles (17), Dyer and Helwig (18), Call *et al.* (19), Fried and Dameshek (20), Thompson (21), Moore and Weider (22), Kracke (4), and others).

The following experiments were carried out to test the hypothesis that leucocidin-producing organisms, restrained from active invasion of the tissues but so situated that their diffusible toxic products can be absorbed, are capable of producing granulopenia.

EXPERIMENTAL

Method.—Four kinds of organisms were used, *Staph. aureus*, *Strep. haemolyticus*, *Strep. viridans*, and a *Proteus* species, to be described elsewhere, which was isolated from the blood, and post mortem from the spleen and liver of a case of agranulocytic angina (Schultz' syndrome).

Parchment capsules were prepared as follows: two Schleicher and Schüll diffusion shells No. 579 (16 x 100 mm.) were cut down to a length of 2 cm., sterilized in 70 per cent alcohol, and one fitted over the other to form a closed capsule which was sealed with celloidin. Such a capsule was filled, by means of a sterile syringe and needle, with an 18 hour serum broth culture of the organism to be studied, and the punctures were carefully sealed with celloidin. The outside of the capsule was then thoroughly soaked with 70 per cent alcohol and placed aseptically in the abdominal cavity of a rabbit. The operation consisted of a single incision, just large enough to admit the passage of the capsule, which was carried

relatively little systemic effects because of their pronounced, local injurious effect which fixes them *in situ*. Streptococci, on the contrary, produce far greater systemic effects owing to the invasiveness resulting from their relatively mild local effects. Other examples of a similar nature can be pointed out. The localization of diphtheria bacilli is probably explainable by the accumulation of fibrin which this organism induces in the respiratory mucosa. Nevertheless, the soluble exotoxin formed by these bacilli gradually finds its way into the systemic circulation, very much as the writer has shown to be the case with foreign proteins (11). In view of the studies reported in this and in earlier communications the reason for the disastrous effects that frequently follow surgical interference with a protective inflammatory barrier is obvious.

The fact has been pointed out (1-3) that fixation occurs extremely early in the development of the inflammatory reaction, thus allowing an interval of time for leucocytes to assemble for phagocytosis. This initial phase of fixation walls off the area of injury from the rest of the organism and thus plays a definite part in immunity. The present results suggest that the streptococcal type of inflammation involves so delayed a reaction of fixation that the microorganisms are enabled to disseminate relatively freely.

SUMMARY AND CONCLUSIONS

Trypan blue injected into an area of cutaneous inflammation induced by *Staphylococcus aureus* failed to drain readily to the tributary lymphatics when the dye was injected as early as 1 hour after the inoculation of the microorganisms.

Trypan blue introduced into an area of cutaneous inflammation induced by Pneumococcus Type I was retained *in situ* when the dye was injected about 6 or more hours after the inoculation of the bacteria.

When an area of cutaneous inflammation was induced by the inoculation of a culture of *Streptococcus hemolyticus*, trypan blue injected into it drained readily to the tributary lymphatics for the first 30 hours following the onset of the inflammatory reaction. When the inflammation had lasted for 45 hours or longer, the dye was fixed *in situ* and failed in most instances to reach readily the tributary lymphatics.

The rapidity of fixation of the dye in the instances given would

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TABLE I

Staphylococcus aureus

No. 9-9, operated upon June 22, 1932, 3:30 p.m.

Date	Time	W.B.C.	Eosinophils	Basophils	Neutrophils				Lymphocytes	Large mononuclears
					Myelocytes	Metamyelocytes	Band form*	Segmented		
1932										
June 22	5 p.m.	14,300								
June 23	8 a.m.	23,750		4			3	47	45	1
June 24	a.m.	40,000				1	52	30	17	
June 25	a.m.	18,400		3	2	4	54	22	15	
June 26	a.m.	22,150		7			24	21	38	10
June 27	a.m.	21,350		4			6	2	79	9
June 28	a.m.	21,950		5			2	6	73	14
June 29	a.m.	18,400		7			3	8	73	9
June 30	a.m.	20,200		6		2	5	9	74	4
July 1	a.m.	23,200					2	7	81	10
July 2	a.m.	20,650		2	1	1	6	8	73	11
July 3	a.m.	18,200		2			6	7	76	9
July 4	a.m.	18,350		1			4	13	74	8
July 5	a.m.	16,950					7	9	77	6
July 6	a.m.	14,450				1	5	11	80	3
July 7	a.m.	12,700		1	1	2	8	17	69	3
July 8	a.m.	17,750	1	1			12	12	67	7
July 9	a.m.	17,250		1			4	4	87	4
July 10	a.m.	8,950					8	12	77	2
July 11	a.m.	10,850	1	1			6	11	71	10
July 12	a.m.	15,000	1	1			4	4	77	13
July 13	a.m.	14,750		2			4	9	73	12
July 14	a.m.	15,150		1			2	8	88	1
July 15	a.m.	18,750		2			5	7	75	11
July 16	a.m.	15,500		3			1	10	83	3
July 17	a.m.	13,650		1			5	19	72	3
July 18	a.m.	15,400		3				9	85	3
July 19	a.m.	12,050		3			2	8	83	4
July 20	a.m.	15,550		3			5	12	75	5
July 21	a.m.	13,800	1	6			4	4	81	5
July 22	a.m.	9,400		3			1	9	85	2
July 23	a.m.	9,400		3			2	15	75	5
July 24	a.m.	12,500		2			8	11	71	8
July 26	a.m.	9,750		7			17	23	48	5
July 28	a.m.	10,350		1			7	7	78	7
				7			4	10	74	5

* The band form corresponds to Schilling's "stabkernige Form."

FIG. 4. Lymphatic vessel from an area of cutaneous inflammation induced by *Streptococcus hemolyticus*. The inflammatory reaction is of over 50 hours duration. The lumen is occluded by a dense thrombus containing leucocytes in abundance. Trypan blue injected into the area of inflammation failed to reach the tributary lymphatics (Rabbit 6-42, Table III). $\times 615$.

PLATE 63

FIG. 5. Part of the lumen of a lymphatic vessel showing its occlusion by a coarse fibrinous reticulum taken from an area of *Staphylococcus aureus* inflammation of over 23 hours duration (Rabbit 10-24, Table I). Trypan blue injected into the area was fixed and failed to reach the regional lymphatics. $\times 1500$.

FIG. 6. Lymphatic vessel in an area of streptococcus inflammation of 26 hours and 15 minutes duration. The lumen is dilated and shows only a small area of fibrin deposit. The remaining portion of the lumen is entirely unoccluded. Trypan blue diffused freely to the regional lymphatic nodes (Rabbit 10-11, Table III). $\times 250$.

FIG. 7. Thrombosed lymphatic vessel in a cutaneous area of inflammation induced by *Pneumococcus* Type I. The inflammatory reaction is of about 23 hours duration. The dye injected into this inflamed area failed to reach the tributary lymphatics (Rabbit 10-02, Table II). $\times 283$.

FIG. 8. Lymphatic vessel in an area of streptococcus inflammation of 19 hours and 20 minutes duration. The lumen is dilated and patent. The dye injected into this area diffused freely to the regional lymphatics (Rabbit 10-10, Table III). $\times 283$.

of sharp depression of the neutrophils. The behavior of the leucocytes is shown in Table III. The animal is still living, more than 6 months after operation, and appears to be in good condition.

Rabbit 10-0.—Received 5 cc. of the culture received by No. 5-1. The animal aborted and died 36 hours after operation. The blood picture is shown in Table IV; there was an acute leucopenia. At autopsy, smears and cultures were negative, the bone marrow was practically exhausted of granulopoietic elements. The capsule was intact, and its contents viable.

TABLE II

Staphylococcus aureus

No. 2-3, operated upon Nov. 12, 1932, 4 p.m.

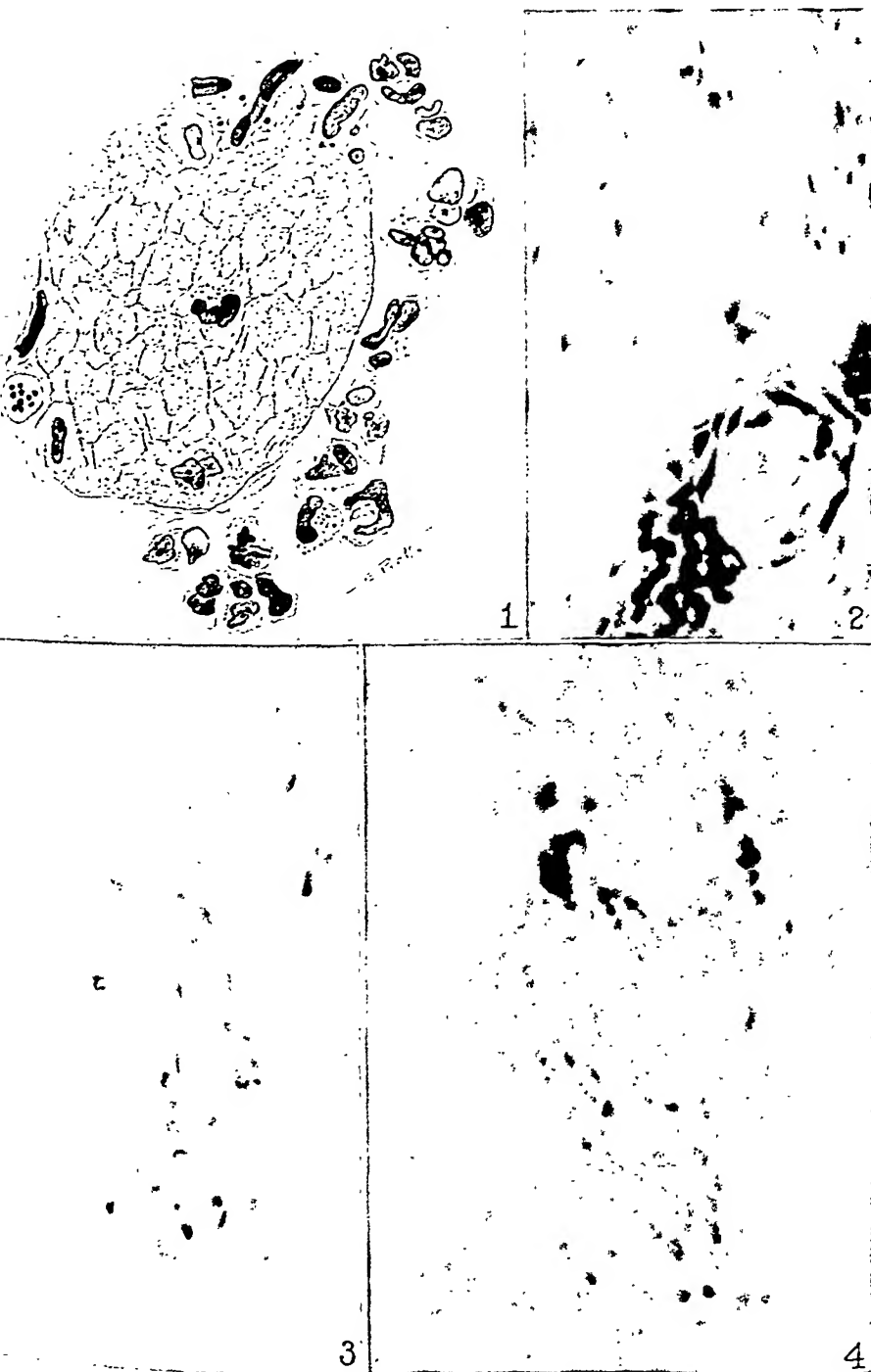
Date	Time	W.B.C.	Eosinophils	Basophils	Neutrophils				Lymphocytes	Large mononuclears
					Myelocytes	Metamyelocytes	Band form	Segmented		
1932										
Nov. 12	5 p.m.	8,550		8			26	48	8	10
Nov. 13	8 a.m.	16,400		3		6	60	14	9	8
Nov. 14	a.m.	10,350	2	6		4	16	5	48	19
Nov. 15	a.m.	7,850	1	7		1	28	19	32	12
Nov. 16	a.m.	13,800		8			12	2	58	20
Nov. 17	a.m.	13,350	2	7		2	34		31	24
Nov. 18	a.m.	25,250	1	8			39	6	34	12
Nov. 19	a.m.	29,850	2			2	18	3	50	23

Animal found dead the morning of the 20th

Streptococcus viridans

Rabbit 10-1.—Received 5 cc. of an 18 hour culture of *Strep. viridans* isolated from a very sore throat. A passage strain was not used as it was practically avirulent for rats and mice, and the intravenous injection of a 24 hour slant culture killed a rabbit only after 5 days. No. 10-1 died 22 hours after operation. There was an acute leucopenia followed by a pneumococcus septicemia (i.e., clinical agranulocytosis). The striking blood picture is shown in Table V. At autopsy, immediately following death, pneumococcus was isolated in pure culture from the heart blood. There was little gross pathology. The bone marrow showed severe destruction of the granular cells (Fig. 3) and a large amount of fat; the erythropoietic elements appeared normal. The capsule was found to be intact and was immediately transferred to another rabbit.

Rabbit 10-2.—Received the capsule from No. 10-1 after it had been thoroughly washed with 70 per cent alcohol. The blood picture is shown in Table VI. The



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TABLE IV

Streptococcus haemolyticus

No. 10-0, operated upon June 22, 1932, 4:30 p.m.

Date	Time	W.B.C.	Eosinophils	Basophils	Neutrophils				Lymphocytes	Large mononuclears
					Myelocytes	Metamyelocytes	Band form	Segmented		
1932										
June 22	5 p.m.	9,000	2	11						
June 22	6 p.m.	8,350	1	2			10	16	61	
June 22	7 p.m.	6,100		7			15	16	66	
June 22	8 p.m.	15,300		2			26	17	50	
June 22	9 p.m.	8,250				5	27	29	37	
June 22	10 p.m.	7,500	1	3		1	30	25	44	
June 22	12 m.	9,750		5		1	29	39	27	
June 23	2 a.m.	6,100	1	2	1		32	38	24	
June 23	3 a.m.	6,000			1		38	23	35	
June 23	8 a.m.	3,600		5			37	32	31	
June 23	12 m.	3,800				3	24	33	34	1
June 23	4 p.m.	2,750		9		8	29	20	43	
					1	36	11	41	2	

Animal aborted and died

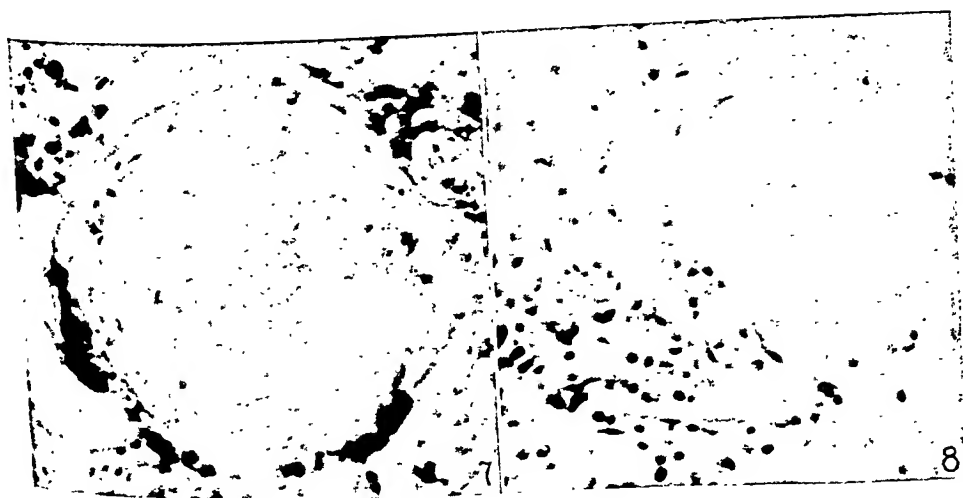
TABLE V

Streptococcus viridans

No. 10-1, operated upon July 28, 1932, 5 p.m.

Date	Time	W.B.C.	Eosinophils	Basophils	Neutrophils				Lymphocytes	Large mononuclears
					Myelocytes	Metamyelocytes	Band form	Segmented		
1932										
July 28	6 p.m.	7,250		10						
July 29	8 a.m.	3,550	1	24			13	44	28	5
July 29	12 m.	1,650	2	4		1	9	8	54	3
July 29	1 p.m.	1,500	1	5	5	3	6		83	2
July 29	2 p.m.	1,250	1	2	3	1	14	1	64	9
July 29	3 p.m.	900	1	2		5	13	6	68	2
							1		88	8

Animal died at 3:30 p.m.



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TABLE VII

Proteus sp.

No. 9-1, operated upon May 24, 1932.

Date	Time	W.B.C.	Eosinophils	Basophils	Neutrophils				Lymphocytes	Large mononuclears
					Myelocytes	Metamyelocytes	Band form	Segmented		
1932										
May 24	a.m.	7,550								
May 25	a.m.	7,750		3						
May 26	a.m.	16,750	2	5		7	25	26	29	10
May 27	a.m.	11,450	1	6	2	4	15	6	53	13
May 28	a.m.	25,400	1	11		2	5	12	67	7
May 29	a.m.	7,250	1	4	3	11	7	8	36	23
May 30	a.m.	30,000		7			11	3	54	25
May 31	a.m.	20,100		3		3	12	18	55	5
June 1	a.m.	23,950		7		1	14	25	38	19
June 2	a.m.	14,850		5			12	32	37	11
June 3	a.m.	17,750	1	7			7	23	60	5
June 4	a.m.	16,650	1	4		1	5	22	62	2
June 5	a.m.	12,500		8			3	23	62	7
June 6	a.m.	10,850	1	2		1	12	16	60	3
June 7	a.m.	9,750		9			2	4	89	2
June 8	a.m.	8,200		5		4	7	5	73	2
June 9	a.m.	11,350		3		2	6	2	85	
June 10	a.m.	11,700	1	7			5	5	85	2
June 11	a.m.	10,450	2	2			6	5	74	7
June 12	a.m.	11,950	1	5			7		89	
June 13	a.m.	9,250		8			6	5	83	
June 14	a.m.	20,500	1	4				10	78	4
June 15	a.m.	18,150	1	2		13	21	60	1	
June 16	a.m.	10,500		4		28	9	59	1	
June 17	a.m.	9,500	2	9		9	18	68	1	
June 18	a.m.	15,950		6		1	10	76	1	
June 19	a.m.	11,950		3		3	12	79		
June 20	a.m.	9,900		1		2	8	87		
June 21	a.m.	7,600		3		2	6	91		
June 22	a.m.	7,450	1	6		1	5	87	4	
June 23	a.m.	10,650	1	6		2	8	80	3	
June 24	a.m.	11,250	2	5	1	7	9	76		
June 27	a.m.	9,350		5		3	19	71		
June 30	a.m.	10,050		3		11	8	76		
July 2	a.m.	8,650	2	1		2	6	73	14	
July 4	a.m.	11,300	1	2		6	15	63	11	
						6	10	77	6	
						6	34	54	3	

EXPERIMENTAL GRANULOPENIA, DUE TO BACTERIAL TOXINS ELABORATED IN VIVO

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PLATES 64 AND 65

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The data presented in this paper are the outgrowth of an attempt to demonstrate a possible infectious origin of the blood dyscrasia which has been variously designated as "agranulocytosis" (1), "agranulocytic angina" (2), "granulopenia" (3), etc., of which at least 550 cases have been reported. The literature has recently been reviewed by Kracke (4) and others.

The disease appears as a symptom complex with the most significant lesion in the bone marrow and blood, where the supply of neutrophilic leucocytes is depleted and their production is stopped or inhibited. The result is a leucopenia which is characterized by a granulocytopenia and a relative lymphocytosis; the blood platelet and erythrocyte counts are usually unaffected. The elimination of the granulocytes so weakens the defensive properties of the body that invasion of the blood stream by organisms present in the body as saprophytes or in low grade infections is a simple matter, and a fatal sepsis frequently results. The clinical manifestations are largely fortuitous, depending on the nature and behavior of the invading organisms.

If this conception of the nature of the disease is valid, the etiologic factor must be something which has the power to depress the function of the bone marrow, and furthermore it must be specifically toxic for granulocytes.

Arsenicals, benzene, and x-ray are capable of producing the clinical picture of agranulocytosis (4), but there is little evidence that any of them plays an important rôle in the etiology of the steadily increasing number of cases being reported. Attempts to produce the disease in

GRANULOPENIA DUE TO BACTERIAL TOXINS

TABLE VII

Proteus sp.

No. 9-1, operated upon May 24, 1932.

Date	Time	W.B.C.	Eosinophils	Basophils	Neutrophils				Lymphocytes	Large mononuclears
					Myelocytes	Metamyelocytes	Band form	Segmented		
1932										
May 24	a.m.	7,550		3		7	25	26	29	10
May 25	a.m.	7,750	2	5		4	15	6	53	13
May 26	a.m.	16,750	1	6	2	2	5	12	67	7
May 27	a.m.	11,450	1	11	3	11	7	8	36	23
May 28	a.m.	25,400	1	4					54	25
May 29	a.m.	7,250		7			11	3	55	5
May 30	a.m.	30,000		3	3	3	12	18	38	19
May 31	a.m.	20,100		7	1	1	14	25	37	11
June 1	a.m.	23,950		5			12	32	60	5
June 2	a.m.	14,850	1	7			7	23	62	2
June 3	a.m.	17,750	1	4		1	5	22	62	7
June 4	a.m.	16,650		8			3	23	60	3
June 5	a.m.	12,500	1	2	1	12	16	60	89	2
June 6	a.m.	10,850		9		2	4	5	73	2
June 7	a.m.	9,750		5	4	7	5	2	85	2
June 8	a.m.	8,200		3	2	6	2	5	85	7
June 9	a.m.	11,350	1	7		5	5	5	74	4
June 10	a.m.	11,700	2	2		6	5	89	83	1
June 11	a.m.	10,450	1	5		7		83	78	1
June 12	a.m.	11,950		8		6	5	78	60	1
June 13	a.m.	9,250	1	4			10	59	68	1
June 14	a.m.	20,500	1	2		13	21	76	79	1
June 15	a.m.	18,150		4		28	9	87	91	4
June 16	a.m.	10,500	2	9		9	18	80	76	3
June 17	a.m.	9,500		6		1	10	87	80	
June 18	a.m.	15,950		3		3	12	87	76	
June 19	a.m.	11,950		1		2	8	91	76	
June 20	a.m.	9,900		3		2	6	87	76	
June 21	a.m.	7,600	1	6		1	5	87	76	
June 22	a.m.	7,450	1	6		2	8	80	76	
June 23	a.m.	10,650	2	5	1	7	9	76	76	
June 24	a.m.	11,250		5		3	19	71	76	
June 27	a.m.	9,350		5		11	8	76	76	
June 30	a.m.	10,050	2	3		2	6	73	73	14
July 2	a.m.	8,650	1	1		6	15	63	77	11
July 4	a.m.	11,300	1	2		6	10	77	77	6
						6	34	54	54	3

through the skin, abdominal wall, and peritoneum in the midventral line. Ether anesthesia was used. After insertion of the capsule, the wound was sutured and an antiseptic dressing applied.

After the operation, the blood picture was carefully followed by total and differential leucocyte counts made hourly, daily, or twice daily as the condition of the animal and the blood picture indicated. Rabbits which died were carefully autopsied, material fixed for histological study, and the condition of the bone marrow ascertained by impression smears fixed with methyl alcohol and stained with Giemsa's or Leishman's stain. The histopathology will be described separately.

The experiments were controlled by preoperative counts and specifically by the blood pictures of two rabbits which received capsules of sterile serum broth.

The results of typical experiments are given in tabular form. Twenty animals have been used, two of which died of *Strep. haemolyticus* septicemia as the result of leaking capsules. The differential counts were made on blood obtained from the ear veins of the rabbits and stained with Leishman's stain. Schilling's (24) modification of Arneth's classification of the leucocytes was used, except that the large and small lymphocytes were not separated. Schilling's hemogram method of recording the counts proved to be valuable. The figures in the accompanying tables (hemograms) represent percentages determined by a count of at least one hundred cells in all cases where the total counts were above a thousand.

PROTOCOLS

Staphylococcus aureus

Rabbit 9-9.—Received a capsule containing 5 cc. of an 18 hour culture of virulent *Staph. aureus* isolated from a case of chronic furunculosis with slight granulopenia. The behavior of the leucocytes is shown in Table I. There was a chronic granulopenia with an absolute lymphocytosis. The animal died Sept. 20, 6 months after operation. The contents of the capsule were viable and not contaminated. The capsule was imbedded in caseous pus to a depth of 2 cm. Death was due to the peritonitis resulting from fistulation of a loop of the intestine involved in the adherent reaction mass. The terminal infection did not raise the leucocyte count above normal. The granular cells of the bone marrow were degenerative and relatively few in number (Fig. 1).

Rabbit 2-3.—Received a capsule containing 3 cc. of an 18 hour culture of *Staph. aureus* isolated from an abscess and passed through five mice. The blood picture is shown in Table II. The animal died on the 7th day after operation. There was extensive destruction of the pseudoeosinophils in the peripheral blood where the cells were very pathological. The large mononuclears were markedly increased. *Strep. viridans* was isolated from the heart blood and spleen. There was no peritonitis. The smears and cultures of the peritoneal fluid were negative. The bone marrow was depleted but not exhausted of granulopoietic elements. The capsule was intact; contents viable. The histopathology was that of severe intoxication.

Controls

Rabbits 9-4 and 9-7.—Each received a capsule containing 5 cc. of sterile serum broth from the same two lots used in the implants cited above. There was an initial leucocytosis, consequent of the operation, but the count soon returned to slightly above normal and continued at that level. No. 9-4 was sacrificed to determine the condition of the capsule and amount of reaction; No. 9-7 is still living and in good condition. The behavior of the leucocytes in the controls is illustrated in Table VIII.

TABLE VIII

Control

No. 9-7, operated upon June 18, 1932.

Reported upon June 18, 1932.										
Date	Time	W.B.C.	Eosinophils	Basophils	Neutrophils				Lymphocytes	Large mononuclears
					Myelocytes	Metamyelocytes	Band form	Segmented		
1932										
June 18	p.m.	12,250	2	8						
June 19	a.m.	14,200	1	2			27	43	20	
June 20	a.m.	11,400	2				21	37	39	
June 21	a.m.	6,100	3	2			5	32	55	6
June 22	a.m.	7,750	1	2		2	7	30	55	1
June 23	a.m.	9,250		2		1	10	32	54	
June 24	a.m.	16,650	2	1			3	35	59	1
June 25	a.m.	9,150		4			6	60	25	15
June 26	a.m.	7,200		3			4	35	41	16
June 27	a.m.	7,650	1	5			5	40	42	10
June 28	a.m.	6,750	1	4			1	33	52	8
June 29	a.m.	13,550	3	9			5	61	24	5
June 30	a.m.	15,450		10			3	53	28	4
July 1	a.m.	10,050	1	8				38	44	8
July 2	a.m.	8,300	2	6			1	48	34	8
July 3	a.m.	10,050	2	6			4	40	43	5
July 4	a.m.	8,900	1	11			4	40	42	6
July 5	a.m.	9,850		11			3	47	30	8
July 6	a.m.	9,900	3	5			6	40	38	5
July 7	a.m.	10,450		4			2	35	49	6
July 8	a.m.	9,300	4	2			5	38	50	3
July 9	a.m.	11,950	6	3			2	32	55	5
July 10	a.m.	12,300	3	3			1	30	55	5
July 11	a.m.	8,300	5	5			1	37	54	2
July 12	a.m.	10,350	4	5			6	39	36	9
July 13	a.m.	10,500					1	37	49	4
July 14	a.m.	8,850	2	3			4	40	48	8
							5	58	30	2

TABLE I—*Concluded*

Date	Time	W.B.C.	Eosinophils	Basophils	Neutrophils				Lymphocytes	Large mononuclears
					Myelocytes	Metamyelocytes	Band form	Segmented		
1932										
July 30	a.m.	11,950								
Aug. 2	a.m.	9,500		3						
Aug. 3	a.m.	12,900		3						
Aug. 5	a.m.	10,600	1	4			1	12	79	5
Aug. 9	a.m.	8,800	2	1			10	14	65	8
Aug. 12	a.m.	11,350	1	2			5	4	86	
Aug. 15	a.m.	8,550	1	2			5	14	73	5
Aug. 18	a.m.	8,500	1	2			2	10	78	7
Aug. 22	a.m.	8,500	1	2			2	7	84	4
Aug. 25	a.m.	8,500		1			11	16	66	4
Aug. 29	a.m.	8,550		1			5	12	69	13
Aug. 31	a.m.	6,500	1	2			2	4	81	12
Sept. 1	a.m.	6,600		1			8	3	79	8
Sept. 2	a.m.	6,150		2			12	15	69	2
Sept. 3	a.m.	7,300	1				3	6	86	2
Sept. 5	a.m.	8,100	3	2	1		13	7	72	6
Sept. 7	a.m.	6,050	3				3	3	87	2
Sept. 8	a.m.	8,050	3				1	1	94	1
Sept. 9	a.m.	10,550	1				9	6	76	6
Sept. 10	a.m.	6,050	3				2	6	87	4
Sept. 12	a.m.	6,150	7				8	4	80	5
Sept. 13	a.m.	5,750	6				11	8	73	1
Sept. 14	a.m.	9,500	3	4			27	19	39	7
Sept. 15	a.m.	9,500	2	6			11	6	68	7
Sept. 16	a.m.	15,500	3	2			2	2	80	7
Sept. 17	a.m.	11,400	1	1			2	2	90	1
Sept. 19	a.m.	12,200	1	2			11	4	83	
	a.m.	7,700	4	2			30	20	44	3
			2				13	4	75	2
							3	24	66	5

Animal found dead the morning of Sept. 20

Streptococcus haemolyticus

Rabbit 5-1.—Received a capsule containing 3 cc. of an 18 hour culture of hemolytic streptococci isolated from a pelvic abscess and passed through five mice. The total count dropped to 3,300 white blood cells and the animal aborted but survived.¹ The blood picture has shown great variation with occasional periods

¹ Unfortunately two animals (Nos. 5-1 and 10-0), which had not been isolated a sufficient period of time, became mixed with the lot destined for the experiment.

in a relative rather than absolute shift to the left. This shift to the left clearly demonstrated the sustained elaboration and activity of the toxic factor.

In none of the cases presented above was there an abnormal appearance of the erythropoietic elements in the bone marrow, and blood platelets were always abundant in the blood smears. The eosinophils tended to disappear and the basophils to increase with increased intoxication. A sharp drop in the number of neutrophils was usually preceded by a marked basophilia which generally disappeared as the neutropenia developed. The appearance of the large mononuclears varied greatly with different animals. The degree of variation was not correlated with any particular species of parasite, but appeared to be dependent upon the reactivity of the individual host.

A significant phenomenon was observed where there was a secondary infection superimposed upon the toxigenic strain. There was an immediate antagonistic response of the bone marrow with an increase of granulocytes in the peripheral blood, but such compensatory efforts undoubtedly merely hastened the exhaustion of the bone marrow and shortened the life of the animal. This phenomenon has its clinical counterpart in the cases cited by Doan (25), in which the stimulation of myelopoiesis by repeated 1/20 erythema doses of x-rays over the long bones showed a prompt response in the peripheral blood, only to be quickly followed by a relapse, with precipitation of complete aplasia, which was rapidly fatal despite all therapy. Doan's suggestion that "the fatal recurrence in such cases may be simply the development of complete decompensation of the organ following the last heroic effort to respond to stimuli ordinarily effective in initiating and maintaining myelopoiesis" is given experimental support. It is apparent that great caution should be exercised in any therapeutic stimulation of bone marrow with a low myelopoietic reserve.

It appears that the pyogenic bacteria are capable of producing granulocytopenia only when they are restrained from active penetration into the tissues, yet are so situated that their toxic products can be readily absorbed; otherwise they stimulate the leucopoietic system and produce a leucocytosis. Since the injection of filtrates of these organisms has so consistently failed to induce a depression of the granulocytes (4), it appears that the constancy of the supply of toxin

TABLE III
Streptococcus haemolyticus
 No. 5-1, operated upon June 22, 1932, 4 p.m.

Date	Time	W.B.C.	Eosinophils	Basophils	Neutrophils				Lymphocytes	Large mononuclears
					Myelocytes	Metamyelocytes	Band form	Segmented		
1932										
June 22	5 p.m.	5,200	2	8						
June 23	8 a.m.	5,050	1	6						
June 23	4 p.m.	3,300		3						
June 24	8 a.m.	4,650	1	5						
June 24	12 m.	5,850	1	6						
June 24	4 p.m.	12,450		2						
June 24	6 p.m.	8,200		1						
June 24	8 p.m.	19,400		13						
June 25	10 p.m.	39,450		8						
June 25	12:45 a.m.	5,250		11						
June 25	1:45 a.m.	6,950	1	6						
June 25	8 a.m.	9,700	2	4						
June 25	4 p.m.	16,600		2						
June 26	9 p.m.	7,950		4						
June 26	8 a.m.	24,000		4						
June 27	5 p.m.	7,300		2						
June 28	8 a.m.	19,550	2	8						
June 29	8 a.m.	10,650		5						
June 30	8 a.m.	25,500		10						
July 1	8 a.m.	17,900		16						
July 2	8 a.m.	19,250		10						
July 3	8 a.m.	7,300	1	1						
July 5	8 a.m.	18,600		1						
July 7	8 a.m.	17,800		6						
July 9	8 a.m.	18,200	1	9						
July 11	8 a.m.	17,050	1	5						
July 15	8 a.m.	24,750		3						
July 19	8 a.m.	17,250		1						
July 23	8 a.m.	18,600		2						
July 28	8 a.m.	11,200	1	3						
July 30	8 a.m.	10,700	1	6						
Aug. 4	8 a.m.	12,250		3						
Aug. 10	8 a.m.	12,500		6						
Aug. 13	8 a.m.	6,150		10						
Aug. 19	8 a.m.	8,950	2	6						
Aug. 26	8 a.m.	4,000	1	6						
Sept. 1	8 a.m.	6,000		3						
		7,550		7						

Additional counts show no further differences in behavior

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EXPLANATION OF PLATES

PLATE 64

- FIG. 1. Bone marrow of Rabbit 9-9. Impression preparation, Leishman's stain. Note the disintegrating myeloid cells, and the failure of their nuclei to stain. Erythropoietic elements normal and well stained. $\times 1,100$.
- FIG. 2. Extreme myeloid aplasia of the bone marrow due to *Strep. viridans* toxin. Erythropoietic elements normal. Impression preparation, Leishman's stain. $\times 1,100$.
- FIG. 3. Disintegration of myeloid cells, and large amount of fat in the rib marrow. Action of *Strep. viridans*, but the course was more acute and exhaustion less complete than in the case of Fig. 2. Leishman's stain. $\times 1,100$.

PLATE 65

- FIG. 4. Pathological neutrophils in the peripheral blood of Rabbit 9-9 just before the onset of the granulopenia. Leishman's stain. $\times 2,200$.
- FIG. 5. Vacuolization of the cytoplasm of a young band form (or late meta-myelocyte) due to the action of *Strep. viridans* toxin. Blood smear stained with Leishman. Note the disintegrating band and normal platelets in the upper left corner. $\times 2,200$.
- FIG. 6. Pathological band forms in the peripheral blood of a rabbit that received *Strep. haemolyticus*. Leishman's stain. $\times 2,200$.

depression of the leucocytes was immediate and such neutrophils as could be found were in various stages of disintegration. The animal died 17 hours after operation. Cultures of the heart blood taken just before death yielded pneumococcus. At autopsy the capsule was found to be intact and its contents viable. The bone marrow showed as advanced cellular pathology as did that of No. 10-1, but the granular cells were more numerous.

TABLE VI
Streptococcus viridans
No. 10-2, operated upon July 29, 1932, 5 p.m.

Date	Time	W.B.C.	Eosinophils	Basophils	Neutrophils				Lymphocytes	Large mononuclears
					Myelocytes	Metamyelocytes	Band form	Segmented		
1932										
July 29	6:30 p.m.	8,100	2	3						
July 29	9:30 p.m.	1,850								
July 29	10:30 p.m.	700		2			2	26	67	
July 30	11:30 p.m.	450					13	13	71	
July 30	12:30 a.m.	2,100	1	6			6		94	1
July 30	1:30 a.m.	3,350		4			12		80	
July 30	2:30 a.m.	4,700		6	1		28		64	2
July 30	3:30 a.m.	4,100			2		28		64	
July 30	4:30 a.m.	2,000		6	1		16	2	80	
July 30	5:30 a.m.	1,800		1			6		88	
July 30	6:30 a.m.	1,300		4	4		10		84	
July 30	7:30 a.m.	1,650			1		16		76	1
July 30	8:30 a.m.	900	1	2			4		94	2
July 30	9:30 a.m.	3,400		4			6		90	2
July 30	10:30 a.m.	2,100		14	4		10	2	83	2
				4	8		27	2	49	
							6		80	3

Animal died at 10:45 a.m.

These results, with *Strep. viridans*, have been duplicated with two additional rabbits for which the data are omitted to conserve space. One died 12 hours after operation with a total count of 1,400 leucocytes, and the other died 28 hours after operation with a total count of 750 white cells. Both animals had a terminal pneumococcus septicemia.

Proteus sp.
Rabbit 9-1.—Received 5 cc. of an 18 hour culture of a species of *Proteus* (*P. stuartii* var.?) isolated from a fatal case of agranulocytic angina. The behavior of the leucocytes is illustrated in Table VII. There was a chronic granulocyto-

TABLE VII—*Concluded*

Date	Time	W.B.C.	Eosinophils	Basophils	Neutrophils				Lymphocytes	Large mononuclears
					Myelocytes	Metamyelocytes	Band forms	Segmented		
1932										
July 6	a.m.	12,550								
July 10	a.m.	9,000		5						
July 14	a.m.	11,400	1	3			5	9	79	2
July 18	a.m.	10,650	1	2			4	9	77	6
July 22	a.m.	12,800	2	3			6	8	74	9
July 26	a.m.	14,450	2	9			3	15	71	6
July 30	a.m.	17,750	1				20	8	43	18
Aug. 4	a.m.	12,750		7			8	2	83	6
Aug. 8	a.m.	20,000	2	2			17	8	58	10
Aug. 12	a.m.	14,950	1	3			9	7	82	
Aug. 17	a.m.	6,500		3			20	4	70	2
Aug. 18	a.m.	6,900					16	2	77	2
Aug. 19	a.m.	7,900	2	4				1	96	3
Aug. 20	a.m.	11,650	2	2			9		81	4
Aug. 23	a.m.	9,300	1	1			10	2	77	7
Aug. 27	a.m.	9,300	2	1			3	4	88	3
Aug. 31	a.m.	8,050	1	4			2	5	88	2
Sept. 5	a.m.	11,450		3			12	6	71	6
Sept. 9	a.m.	9,300	1	2			4	6	78	9
Sept. 14	a.m.	11,250	4	5			7	4	85	1
Sept. 20	a.m.	10,450	2	1			18	12	53	8
Sept. 27	a.m.	8,750	2				3	5	88	1
Oct. 4	a.m.	10,900		7			2	4	88	4
Oct. 12	a.m.	9,300		2			15	12	68	6
Oct. 18	a.m.	10,850	2				11	12	69	6
Oct. 26	a.m.	10,250					19	6	66	7
Nov. 1	a.m.	8,000	4				5	4	87	2
Nov. 5	a.m.	6,550	3				16	5	70	5
Nov. 8	a.m.	3,000*	1				15	17	59	6
		350†	17	1			15	14	65	4
			7	1	22		12	15	2	20
					9			5	49	17

* Heart blood a few seconds before death.

† Peripheral blood immediately before death.

penia. The animal died 7 months after operation, death resulting from a pneumococcus septicemia. At autopsy the spleen was markedly atrophied, measuring but 2.5×13 mm. The bone marrow showed but a few myelocytes and premature band forms. There was no reaction mass about the capsule, as in all other cases, the capsule being covered by a thin connective tissue membrane rather than being increased in a mass of pus. The capsule was intact, and its contents viable.



DISCUSSION

Staphylococcus aureus, *Streptococcus haemolyticus*, and a species of *Proteus* each produced a more or less chronic granulocytopenia which terminated with a fatal infection by a different bacterial species. *Streptococcus viridans* showed surprising virulence, producing an acute leucopenia within a few hours. The picture produced in rabbits by *Streptococcus viridans* toxin was analogous with that of acute agranulocytosis in man. *Streptococcus viridans* showed a virulence under the experimental conditions that was not manifested by direct animal inoculation, and the toxicity for the leucopoietic system in general was more pronounced than in any of the other cases.

The pathogenic factor was a diffusible toxic substance, and, although the writer has found no mention of the diffusibility of leucocidin, the specificity of its action indicates that it was leucocidin with which we were dealing. Furthermore, filtrates of the cultures used in the capsules brought about the disintegration of leucocytes when mixed with a leucocytic suspension and observed under the microscope.

It is of interest that the production of lymphocytes was not inhibited, and in some cases (*Staphylococcus aureus*) actually stimulated. Even in the case of *Streptococcus viridans*, in which the lymphocytes and large mononuclears were reduced in number, there were no pathological changes in their cellular structure and their depletion was probably due to the severity of the shock to the leucopoietic system. On the other hand, the depletion of the granulocytes was inaugurated by a visible disintegration of the granulocytes in the peripheral blood which began immediately after implantation of the cultures. The hemograms do not give an adequate conception of the degree of destruction of the neutrophils as, with few exceptions, the recorded neutrophils were pathological (Figs. 4 to 6). The action of the toxin was shown by excessive lobulation and vacuolization of the nuclei, vacuolization and loss of outline of the cytoplasm, and swelling and loss of the pseudoeosinophilic granules.

In the chronic cases (Nos. 9-1, 9-9, 5-1), the neutropenia appeared to be primarily due to the destruction of the polynuclear cells. The percentage of band forms, except for intermittent increases, was but little above normal, which in the absence of the polynuclears, resulted

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is a highly important factor in the production of a detectable leucopenia. These conditions are satisfied in a chronic focal infection.

Our conception of the usual origin of the clinical agranulocytic syndrome is that there is a primary focus of infection by one of the leucocidin-producing organisms from which leucocidin is diffused into the blood stream where it affects the neutrophils, and if present in sufficient quantity and toxicity it injures the granulopoietic elements of the bone marrow. With the removal of the granulocytes and the cutting off of the supply, the body is liable to invasion and a fatal sepsis frequently results.

CONCLUSIONS

Pyogenic organisms, under conditions simulating a focal infection, are capable of producing in rabbits a granulopenia which may allow generalized infection and death. It is suggested that agranulocytosis in man is due to the action of leucocidin, rather than to a specific microorganism.

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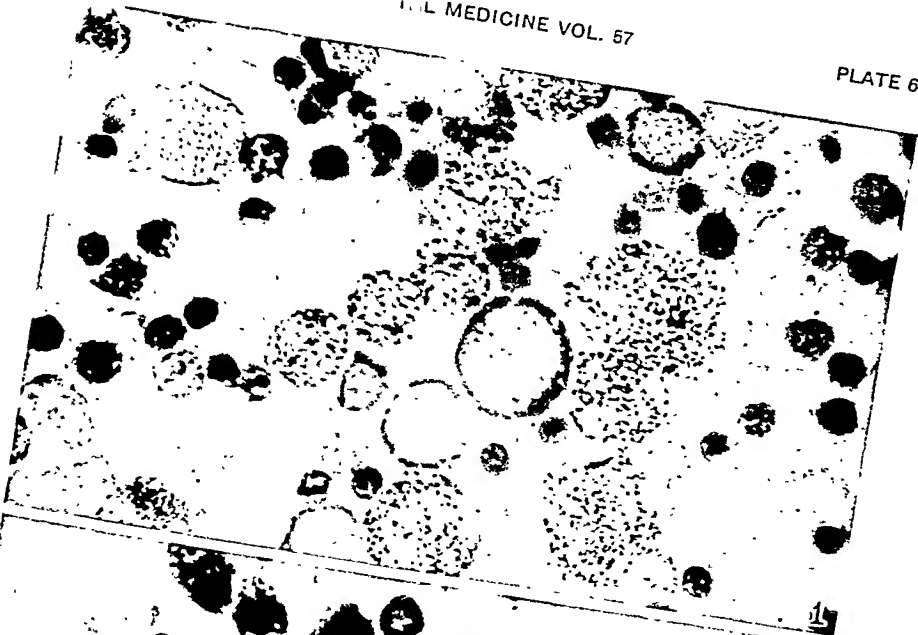
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CONCLUSIONS

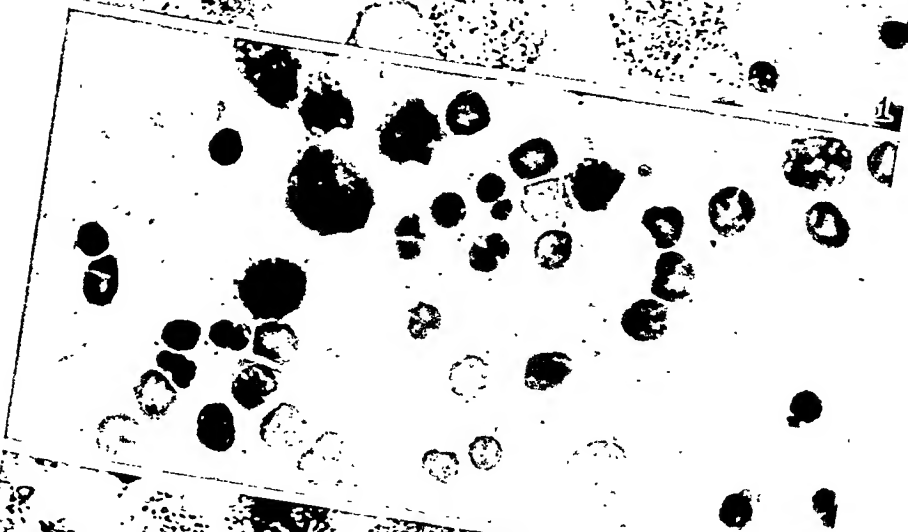
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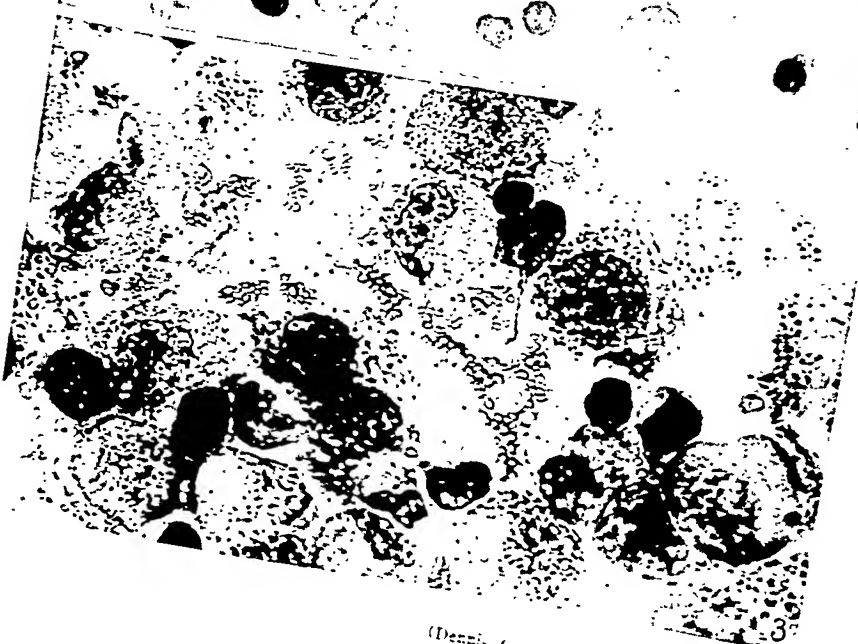
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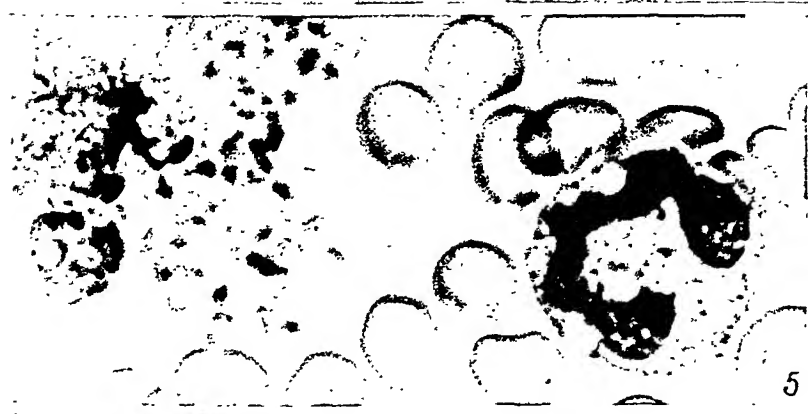
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